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A study of the histozoic oyster parasite, Perkinsus marinus: I. Disease processes in American oysters (Crassostrea virginica). II. Biochemistry of Perkinsus marinus

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A STUDY OF THE HISTOZOIC OYSTER PARASITE, *PERKINSUS MARINUS*: I) DISEASE PROCESSES IN AMERICAN OYSTERS (*CRASSOSTREA VIRGINICA*); II) BIOCHEMISTRY OF *PERKINSUS MARINUS*

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APPROVAL SHEET

This dissertation is submitted in partial fulfillment of the requirements for the degree of

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DEDICATION

This dissertation is dedicated to my parents Mrs V. Sobha Rani, Mr. Nagabhushana Rao, and to Dr. Suseela Botlagudur -my aunt, my mother away from home, my role model and mentor.

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ABSTRACT

Numerous studies have investigated the disease processes of the oyster, *Crassostrea virginica* parasite, *Perkinsus marinus*. However, *P. marinus* disease processes and transmission dynamics are still a matter of speculation and not well understood. The objectives of this study were to investigate: 1) the most effective lifestage of *P. marinus* in initiating infection, 2) the synergetic effects of temperature, salinity and *P. marinus* cell dosage on disease prevalence, 3) suppression of host reactive oxygen intermediates (ROI) by *P. marinus*, 4) the localization of acid phosphatase (AP) in the parasite, 5) the effects of temperature and osmolality on parasite AP secretion, and 6) the lipid and fatty acid composition of *P. marinus*.

Infectivity of meront and prezoosporangia stages of *P. marinus* were compared in eastern oysters. Meronts were more infective than prezoosporangia, and the infection was dose dependent. The minimum number of *P. marinus* cells required to initiate infection in oysters was 10^2 cells.

The synergetic effects of temperature, salinity and dose of *P. marinus* cells were examined by exposing oysters to combinations of 3 temperatures, 3 salinities and 2 doses of meronts. Increased prevalence and intensity of infection occurred at high temperatures and salinities, and there was a dose dependent response to infective particles. Temperature was the most important factor influencing the susceptibility to *P. marinus* and subsequent disease development in oysters followed by dose of infective cells and salinity. The effect of interaction of these three factors on disease prevalence was insignificant. However, the interaction of temperature and salinity; and temperature and dose intensified the infection. Temperature and salinity significantly affected the host cellular and humoral factors.

Ability of *P. marinus* cells to suppress/inhibit the host ROI was examined by exposing zymosan-stimulated hemocytes to heat killed- or live-*P. marinus*. *P. marinus* suppression of hemocyte ROI was dose dependent. Suppression of ROI production by heat-killed *P. marinus* was significantly less than live-*P. marinus*. Similarly ROI of hemocytes was reduced, although insignificantly, when zymosan-stimulated hemocytes were exposed to estuarine water preincubated with *P. marinus*. Results suggest that *P. marinus* cells, or their extra-cellular products suppress host ROI production, thus evading this component of the hosts' defense.

Intracellular AP activity in meront and prezoosporangia stages were compared. The effect of temperature and osmolality on AP secretion *in vitro*, by *P. marinus* was also investigated. AP activity in *P. marinus* cells increased with increase in temperature. Meronts had higher AP activity than prezoosporangia. The extracellular AP secretion by *P. marinus* was dose dependent and increased with temperature and

osmolality. Other antioxidant enzymes, catalase, superoxide dismutase, and glutathione peroxidase were not detected in *P. marinus*. Ultrastructural localization of AP in *P. marinus* using electron microscopy revealed that AP activity was primarily in the nucleus, but was also present in the cell membrane. AP may aid the parasite in nutrition and in escaping from the host defense.

To determine the role of lipids and fatty acids in the parasites' development, lipid and fatty acid composition was characterized in meront and prezoosporangia. Differences in lipid classes were observed in meronts cultured in different media. Phospholipids were the major lipid class in meronts while triacylglycerols were the major lipid class in prezoosporangia isolated from infected oyster tissue. Results indicate that meronts may convert wax/cholesterol esters to other lipid classes. Meronts and prezoosporangia had much higher level of arachidonic acid (20:4n-6) than the host. *P. marinus* may actively assimilate arachidonic acid and/or modify short chain fatty acids of the n-6 family from the host.

A study of the histozoic oyster parasite, *Perkinsus marinus* I) Disease Processes in American Oysters (*Crassostrea virginica*)II) Biochemistry of *Perkinsus marinus*

CHAPTER 1

GENERAL INTRODUCTION

The economic and ecological value of the American oyster, *Crassostrea virginica*:

American oyster, *Crassostrea virginica* has traditionally supported a viable commercial fishery. Apart from being a commercially important species, oysters improve the biodiversity of the habitat by forming reefs which offer complex habitat for organisms to thrive on. Oyster reefs render food and shelter to other commercially important species such as weak fish, red fish, blue crab etc. More importantly, oysters act as biological filters, filtering as much as 34 liters of water per hour (Galstoff 1964). With this filtration efficiency, they filter sediment, organic detritus, and pollutants from the water column, rendering it clean (Bahr and Lanier 1981, Newell 1988) and depositing organic matter which in turn is used by benthic organisms inhabiting oyster reefs (Sornin et al. 1983).

The American oyster (Eastern oyster) *Crassostrea virginica* is found along the Atlantic coasts from Gulf of St. Lawrence, Canada, to the Gulf of Mexico, extending all the way to Venezuela (Stanley and Sellers, 1986). Chesapeake Bay oyster fisheries harvested nearly seven million bushels per year during 1835 to 1891 (Brooks 1981 in Haven et al 1978) and as many as 20 million bushels per year between 1875 and 1885. A decline in oyster populations started in 1913. Oyster production declined to 2.4 million bushels per year by 1932,

and had stabilized at about 4 million bushels by 1959. However, a more drastic decline in oyster populations on the east coast of the United States has been observed in since 1960. Oyster landings in Virginia were a mere 45,000 bushels in 1992 (Virginia Marine Resource Commission). Delaware Bay oyster landings declined from 1.2 million bushels in 1950 to 1,000 bushels in 1988.

The impact of the decline in oyster populations is also of an ecological concern. It was estimated that oyster populations in the Chesapeake Bay prior to 1870 filtered the volume of water equivalent to the entire bay in just three days, while it would take more than 300 days for the existing oyster populations (Newell 1988) to filter the same amount of water. Newell (1988) hypothesized that the decline in oyster populations has led to eutrophication and increased phytoplankton biomass resulting in low oxygen levels in the Chesapeake Bay. Although overfishing has been cited as one of the primary reasons for the decline in oyster populations, other factors, such as fresh water kills, diseases, and predation have also contributed to the decline (Hargis and Haven 1988). Within the last 40 years, the decrease of the oyster fishery has been exacerbated by diseases caused by *Perkinsus marinus* (Dermo) and *Haplosporidium nelsoni* (MSX). Previously, disease pressure caused by MSX has been more intense on the oysters than has *P.marinus*. However, since

1986, mortality caused by *P.marinus* has increased dramatically. Now *P.marinus* is considered more important as an oyster pathogen than MSX in the lower Chesapeake Bay (Burreson 1989).

Discovery of *Perkinsus marinus* and taxonomy:

Investigations of recurring oyster mortalities in the Gulf of Mexico during the 1940's resulted in the discovery of the causative agent, a parasite now known as *Perkinsus marinus* (Mackin et al. 1950). At the time of discovery, the disease organism was thought to have fungal affinities and was termed *Dermocystidium marinum*. In 1966, Mackin and Ray (1966) re-assigned *Dermocystidium marinum* to *Labrynthomyxa marina*. The taxonomy of the disease organism has been a debatable subject for many years (Sprague 1954, Mackin and Boswell 1956, Perkins and Menzel 1966, Perkins 1974). Perkins (1974) found that infective cells in oyster tissue, after incubation in thioglycollate medium, enlarge and develop to a cell stage, prezoosporangia (or hypnospores). Upon incubation in sea water, hypnospores produce free swimming biflagellated zoospores. It was hypothesized (Perkins 1974) that zoospores, upon entering the oyster tissue, will develop to meront stage which is the usual form of cell stage noted in infected oysters. Electron microscopic studies of zoospore revealed that it posses an apical complex, a morphological structure, similar to those seen in Apicomplexans. Thus, Levine (1978)

reclassified the disease organism in the Phylum Apicomplexa, Class Perkinsasida, and named the organism, *Perkinsus marinus*. The appropriate taxonomic position of *Perkinsus marinus* is still not agreed upon (Levine 1988, Wolters 1991, Cox 1991, Goggin and Barker 1993, Fong et al. 1993).

Distribution:

P. marinus affects oysters along the Atlantic and Gulf coasts of the United States from Massachussets to as far south as Tabasco, Mexico (Burreson et al. 1993). *P. marinus* is widely distributed in nearly all estuarine systems of the lower Chesapeake Bay (Andrews 1988, Burreson 1989, 1990). Temperature may be the factor restricting *P. marinus* to the southern waters geographically. Before 1950, no *P. marinus* infection was reported in Delaware Bay. It is beleived that *P. marinus* was imported into Delaware Bay through oyster importation from infected areas (Ford and Haskin 1982). Although, there was a lapse in *P. marinus* infection in that area, it has been epizootic in Delaware Bay since 1990 (Ford 1992). Presently, *P. marinus* infection can be detected as far north as Massachussets (Ford 1992). Historically, low salinity waters served as refuge areas for the oysters from the disease caused by *P. marinus*. To date, *P. marinus* seems to have established itself in low salinity waters (e.g. the upper James and Rappahannock River, Chesapeake Bay) and the oysters therein. A four year drought (1986-1990) and warm

water temperature during the summer and fall are believed to be reasons for the dramatic incursion of *P. marinus* into these areas. These drought conditions allowed *P. marinus* to spread into low salinity areas such as Mobjack Bay. *P. marinus* remained resident after the salinity returned to its normal range, post drought.

Wide host specificity exists in *P. marinus* (Perkins 1994). *P. marinus* infects molluscs including *Tagelus plebeius, Mya arenaria, Crepidula plana, Urosalpinx cinerea,* and *Macoma balthica* (Perkins 1994).

Life cycle:

The morphology, and life cycle was studied by Perkins (1966, 1976). The physiological effects of temperature and salinity on the parasite, *in vitro,* have been examined by Perkins (1976), and Chu and Greene (1989). Three cell stages of *P. marinus* are recognized in oysters: trophozoites (meronts and merozoites), prezoosporangia (schizonts, sporangia, hypnospores) and biflagellated-zoospores. Mature and immature meronts (also called merozoites) are spherical, unicellular and uninucleate with an eccentric vacuole measuring about 2-15µm. Meronts in moribund oyster tissue and cultured in fluid thioglycollate medium can enlarge and develop into uninucleate prezoosporangia. Prezoosporangia are much larger (30-200µm) with a large vacuole encompassing the whole cell, pushing the

cytoplasm and nucleus into a thin layer. When prezoosporangia are incubated in sea water for 4-5 days, zoosporulation occurs by repeated division of cytoplasm and nucleus, releasing free swimming, biflagellated zoospores through a discharge tube (Perkins 1966, 1988, Chu and Greene 1989). All life stages are infective and reproduction is vegetative.

Transmission of the parasite into the host:

Since the discovery of the parasite, studies have been directed to elucidate its transmission processes in oysters. As described earlier, all the three known lifestages of *P. marinus* are infective. The ectoparasitic gastropod, *Boonea impressa* is capable of transmitting *P. marinus* between individual oysters under laboratory conditions (White et al. 1987). Whether involvement of this organism or any other scavengers of oysters as reservoir or transmission agent in the field is a matter of speculation and needs further examination. It is widely accepted that in the northern waters, *P. marinus* infection is transmitted by transport of infective cells into the host through the water (Ray 1954, Andrews and Hewatt 1957, Mackin 1962, Andrews 1988). It is hypothesized that infective cells are purged by the oyster or are disseminated into the water column upon death and decomposition of the oyster. Prezoosporangia released into the sea water, after series of cytokinesis and karyokinesis

form numerous free swimming biflaggellated zoospores. Infective cells of *P. marinus* possibly enter the host body through ingestion while feeding and transported into the body through the gut epithelium. Once the parasite is ingested, very little is known about its fate. All *P. marinus* cell stages are sticky, and may attach to gill and mantle tissues through filtration processes of the oyster. How *P. marinus* enters into the host tissue is not completely known. For example, *P. marinus* cells may be phagocytosed by oyster hemocytes and cross gut epithelium via the migrating hemocytes. (Mackin 1951).

Factors affecting the disease caused by *P. marinus*:

The two environmental factors, temperature and salinity are the most important in regulating the incidience of *P. marinus* in oysters in the field and laboratory.

a) Temperature and salinity:

Due to the distribution of the parasite in warm waters, (Ray 1954, Andrews and Ray 1988, Andrews and Hewatt 1957 and Quick and Mackin 1971), it is hypothesized that temperature is an important environmental factor controlling the distribution of *P. marinus*. Low winter temperatures may be the factor controlling the northern geographic boundaries of *P. marinus*. Epizootics of the parasite display a seasonal periodicity in Chesapeake Bay. Prevalence begins to increase in early spring

after water temperatures consistently exceed 20°C (Andrews and Hewatt, 1957; Andrews 1988). The highest disease prevalence and associated mortalities occur during mid to late summer at temperatures above 25°C (Ray, 1954; Andrews and Hewatt, 1957; Andrews 1988). Oysters grown in high salinity areas (20-30 ppt) experienced higher mortalities caused by *P. marinus*, compared to oysters grown in low salinity areas (8-12 ppt) (Mackin 1951). The relationship between temperature, salinity and *P. marinus* infection in oysters has been well documented in the field (Mackin 1951, Mackin 1956, Andrews and Hewatt 1957, Soniat 1985, Criag et al. 1989, Soniat and Gauthier 1989, Crosby and Roberts 1990, Gauthier et al 1990). As the temperatures and salinities decrease during late fall and winter, the prevalence of infection decreases. Soniat (1985) and Paynter and Burreson (1991) reported a significant relationship between salinity and weighted incidence of *P. marinus* infection in oysters. Crosby and Roberts (1990) found a positive correlation between *P. marinus* infection, high salinity (29.3 - 34.9 ppt) and field temperature. Though the parasite was previously restricted to waters with salinities greater than 15 ppt in Chesapeake Bay (Andrews and Hewatt, 1957), *P. marinus* has now spread to waters of lower (< 10 ppt) salinities (Andrews 1988, Burreson, 1989, 1992).

Laboratory studies have also been conducted to investigate the effect of temperature (Mackin 1951, 1956,

Andrews and Hewatt 1957, Perkins 1966, Ray 1954, Chu and LaPeyre 1993a) on *P. marinus* infection in oysters. Generally, prevalence of *P. marinus* in oysters increases with increase in temperature (Chu and LaPeyre 1993a). Studies by Andrews and Hewatt (1957) showed that the development of *P. marinus* infections are retarded below 15°C. It has been suggested that lower water temperature reduces the metabolic activity of the parasite and enhances the oysters' ability to combat infection (Ray 1954).

Results of laboratory studies on salinity effects (Ragone 1991, Chu et al 1993) supported field observations. Ray (1954) reported that infection in oysters maintained at low salinities (10-13.5 ppt) was delayed when compared to oysters at higher salinities (26-28 ppt). Chu et al (1993) reported that only light infections were observed in oysters maintained at low salinity (3 ppt), while heavy infections were observed in oysters maintained at higher salinities (10 and 20 ppt). Although low salinity treatment of oysters in the laboratory did not eliminate the disease, it significantly reduced the oyster mortality (Ragone and Burreson 1993). It was suggested that the low salinity regulates the physiology of the oyster, thereby influencing the outcome of *P. marinus* infection in oysters (Scott et al 1985). On the other hand, Chu and Greene (1989) and Chu and La Peyre (1991) hypothesized that the virulence of *P. marinus* cells may be higher at higher

salinities.

Perkins (1966), and Chu and Greene (1989) investigated the effect of temperature and salinity on *P. marinus* cultures *in vitro*. Perkins (1976) found salinities from 10-35 ppt to be relatively unimportant as a limiting factor. Similarly, Chu and Greene (1989) reported inhibition of zoosporulation at salinities below 6 ppt and temperatures below 28°C.

Most of the above studies have investigated the effect of either temperature or salinity separately on the infection due to *P. marinus*. However, Soniat (1985) and Soniat and Gauthier (1989) have investigated the interaction of temperature and salinity in the field. The product of temperature and salinity described as the interaction term, was closely correlated with the intensity of infection in oysters than was temperature and salinity alone. In a seperate study, Soniat and Gauthier (1989) found correlation between weighted incidence and salinity, but no correlation between the interaction of temperature and salinity was detected. Lack of variability in temperature in the Gulf of Mexico during the sampling period in the field was cited as the reason for absence of correlation between infection intensity in oysters and temperature. Since both the studies were conducted in the field, the variables could not be adequately controlled. In the field, there are numerous variables which cannot be

adequately controlled, and may interact with each other, thereby influencing the outcome of the result. Laboratory studies with proper controls are necessary to establish cause and effect relationships. Only one laboratory study (Fisher et al. 1992) has been conducted to examine the Dermo disease progression and survival in oysters at different temperatures and salinities. They have concluded that temperature is more important than salinity in influencing *P. marinus* infections in oysters. Only the in vitro *P. marinus* culture studies by Perkins (1976); and Chu and Greene (1989) have addressed the synergistic effects of temperature and salinity on the parasites themselves. Laboratory experiments are needed to investigate the interaction of temperature and salinity on disease processes of *P. marinus* and their effect on the physiology of oysters. The interaction of temperature and salinity may alter the outcome of the Dermo infection in oysters than either temperature or salinity alone.

b) Dose and life stage of *P.marinus* cells:

Mackin (1956) suggested that the correlation between disease level and salinity is not a result of a limiting physiological effect on host or parasite, but due to the dilution of waterborne infective particles by fresh water flow into the estuary. Thus, Mackin proposed that "dosage" of the infective particles is more important than the salinity effect. However, Scott et. al. (1985) suggested that

physiological differences in oysters caused by the exposure to different salinities, rather than the dose of the infective particles, were related to the survival of the oysters. Several attempts have been made to determine the dosage and duration required to initiate infection. Mackin (1962) injected different doses (10 - 10^6 cells) of *P. marinus* cells in tissue homogenate of infected oysters (previuosly incubated in thioglycollate medium for 24 hours) into the shell cavity of the oysters. His results indicated that a dose of $5x10^2$ meronts was required to produce substantial mortality from *P. marinus* infection. However, Mackin's (1962) study monitored only the end point, i.e mortality of oysters caused by the pathogen, but not the infection rate or intensity established in oysters. Moreover, only responses of oysters to different dosages of meronts were studied. The response of oysters to different doses of prezoosporangia or zoospores need to be investigated. Results of our preliminary study indicated that prevalence and intensity of *P. marinus* infection are well correlated with the doses of injected meronts. At 23.0 ± 1.6°C and 18.6± 0.6 ppt, the oysters inoculated with a dose of 10^2, 10^3, and 10^4 meronts per oyster, produced 5, 10 and 43% *P. marinus* prevalence respectively; at the same salinity, but at a 2°C higher temperature ie., 25.1 ± 2.5°C, prevalence increased to 34, 52 and 85% (Chu et al., unpublished data). Since both experiments were not conducted at the same time, it is not clear whether the variation of *P. marinus* prevalence

between experiments was caused by the slight increase of temperature or other factors such as the source of meronts. In another preliminary study (Chu unpublished results), when oysters were inoculated with 10^2, 10^3, and 10^4 prezoosporangia/oyster and incubated at a temperature of 26 ± 1.6°C and a salinity of 17.5 ± 1.13 ppt for six weeks, 93, 73, and 54% prevalence occurred. Infection rates in oysters did not appear to be correlated with the concentrations of prezoosporangia injected into the oysters. The above data suggest that: 1) in addition to dose of *P. marinus* cells, temperature may also be an important factor affecting infection rate; and 2) infectivity of *P. marinus* may vary with cell type. Information on the minimal dose required for each infective life stage (i.e. meronts, prezoosporangia and zoospores) of *P. marinus* to initiate infection is also unknown.

It is still unclear as to which life stage is more important as the primary agent in disease transmission. Perkins (1988) observed that infections were easily obtained by exposing oysters to *P. marinus* infected minced tissues containing meronts, and thus suggested that meronts maybe the common agent for disease transmission. Since only very light infections were obtained when eastern oysters were exposed to 10^6 zoospores, Perkins (1988), also concluded that zoospores may not be the most important infective cell type in the life

cycle of the pathogen.

However, one cannot exclude the following possible circumstances. When prezoosporangia are discharged from the disintegrated infected gapers into sea water, millions of zoospores are produced *in vitro* if the temperatures are favourable for sporulation (Perkins 1976, Chu and Greene 1989). Zoosporulation may also be occurring in nature. Prezoosporangia can also withstand temperatures as low as 4°C and sporulation was observed in prezoosporangia previously incubated at 4°C after they are transferred to water at 28°C. Unlike the trophozoites which are passive and dependent on water currents or flushing to be transmitted between oysters, flagellated zoospores are very active and motile and can survive for more than three days in sea water (Perkins 1976, Chu and Greene 1989). Although recent efforts in our laboratory, as well as many other laboratories to zoosporulate prezoosporangia were not successful, biflagellated-zoospores could also be important for disease transmission.

In a recent study, the infectivity of meronts, prezoosporangia and zoospores were investigated by Chu et al. (unpublished results). It was found that when oysters were injected with 10^6 trophozoites, 10^4 prezoosporangia and 10^5 zoospores, infections were detected after 14, 37 and 8 days respectively (Chu et al., unpublished results). Although the

above studies were not conducted at the same time and the same concentration of *P. marinus* infective particles were not used, prezoosporangia and biflagellated zoospores cannot be ruled out as important agents for disease transmission. The infectivity of lifestages, trophozoites, prezoosporangia and zoospores could be very different.

Some studies have been conducted on the host defense against *P. marinus* and *H. nelsoni* (Fisher and Newell 1986, Fisher 1988, Fisher et al. 1989, Chu and La Peyre 1989). Effects of infection on reproduction and physiological status of the organism (Newell 1985, Barber et al. 1988, Ford and Figueras 1988, Chu et al 1993, Chu and LaPeyre 1993a, 1993b) have been examined by the previous investigators. The morphology and life cycle of *P. marinus* (Mackins and Boswell 1956, Perkins 1966, Perkins and Menzel 1976) have also been described. However, gaps still exist in the life cycle of the parasite. To date *P. marinus* disease processes and its transmission dynamics are largely a matter of speculation and still not completely understood.

It is not known which lifestage is more important in disease transmission. Also, the minimum number of infective particles or dose of infective particles required to initiate an infection in the oysters is not known. Synergetic effects of temperature, salinity on the response of oysters to

different doses of *P. marinus* has not been examined. This information is vital in modelling studies and effective management strategies.

Host defense factors:

Hemocytes are the primary line of defense in oysters (Fisher and Newell 1986, Feng 1988). Hemocyte structure and functions have been reviewed by Cheng (1984) and Fisher (1986). Hemocyte defense functions include inflammation, phagocytosis, and encapsulation. Some defense functions of *C. Virginica* and *C. gigas* have been compared by La Peyre (1993). Molluscan hemocytes are not only able to recognize a wide variety of foreign particle and organisms, but are also able to readily phagocytose them *in vivo* and *in vitro* (Tripp 1960, Cheng 1975, Feng 1988). Apart from the above mentioned functions, oyster hemocytes are also able to release toxic oxygen metabolites and lysosomal enzymes which are microbicidal in function (Cheng 1984, Wishkowsky 1988, Adema et al. 1991). Lysosomal enzymes such as lysozyme(s), acid phosphatase, beta-glucuronidase, lipase and aminopeptidase have been identified in the hemocytes of bivalves including oysters (Cheng and Rodrick 1975, Cheng 1976, Yoshino and Cheng 1976, Mohandas and Cheng 1985, Chagot 1989, Pipe 1990). Lysozymes are involved in bacteriolysis, opsonization, anti-viral and neoplastic activity (Jolles and Jolles 1984, Lie and Syed 1986), digestive functions (Dobson et al. 1984).

Lysozyme activity in bivalves has been described before (Mc Henry and Birberk 1982, Mc Dade and Tripp 1967a, 1967b, Cheng and Rodrick 1974, Hardy et al 1976, Hawkins et al. 1993).

Evasive mechanisms of *P. marinus:*

In a host-parasite relationship, the success or failure of the parasite in establishing infection in the host depends upon the effectiveness of the internal defense system of the host to eliminate the invading parasite and the ability of the parasite to evade the host defense. It is generally accepted that immunocompetency of the host and infectivity of the parasite are governed by genetic factors and mediated by extrinsic environmental factors. Various host cellular and humoral defense factors, in both vertebrates and invertebrates, contribute to extracellular and intracellular lysosomal hydrolases, agglutinnation by agglutinnins, and hemolysis by hemolysin. Cellular factors include encapsulation, and phagocytosis. Oxidative burst, which is triggered by phagocytosis and activation of phagocytes, resulting in the production of toxic oxidative radicals, is particularly important. When macrophages/hemocytes are stimulated by foreign particles or organisms, stimulation of NADPH oxidase (Takeshige and Minakami 1987, Jones et al. 1982) and activation of the hexose monophosphate pathway occurs. This process is accompanied by production of toxic reactive oxygen intermediates such as $^1O_2$, O_2^-, OH^-, and H_2O_2 which may

be involved in cellular killing. This process is called "respiratory burst". This process is also accompanied with the emission of light, "chemiluminescence". Also, H_2O_2, along with myeloperoxidase and halide ions, results in the formation of hypohalites and singlet oxygen, which are microbicidal (Chung and Secombes 1988, Schlenk et al. 1991). Involvement of myeloperoxidase in the respiratory burst of *C. virginica* hemocytes has been reported (Austin and Paynter, 1994/5).

To survive, proliferate and ultimately establish infection within the host, the parasite itself displays a number of elaborate strategies that enable evasion of the host's defense system during all stages of immune response. Several mechanisms have been described for protozoan parasites of the genera, *Leishmania, Toxoplasma, Trypanosoma* and *Plasmodium* spp. These include, but not limited to, i) facilitate invasion and aquire nutrients for growth through secretion of lipolytic and proteolytic enzymes, ii) scavenge/inhibit respiratory burst or destroy toxic compounds generated from phagocyte oxidative metabolism, iii) withstand or circumvent the hydrolytic activity of lysosomes, and iv) modulate the T-cell response (Pino-Hess et al. 1985, Bogdan et al. 1990, Hall and Joiner 1991). These mechanisms play a significant role in the parasites' pathogenecity. Particularly critical is the capacity of certain parasites to

subvert the lethal effects of the oxidative burst (Mauel 1984, Bogdan et al. 1990, Mkoji et al. 1988a, 1988b, Nare et al. 1990, Connors et al. 1991, Mori and Hokoyama 1993).

It has been documented that certain protozoan parasites (*Leishmania*, *Toxoplasma*, and *Trypnosoma* spp) have the capacity to be phagocytosed without stimulating a respiratory burst and to escape superoxide dependent killing by secreting anti-oxidant enzymes such as catalase, superoxide dismutase (Weiss et al. 1987), acid phosphatase (Remaley et al. 1984, Le Gall et al. 1991), cytochrome C peroxidase, glutathione peroxidase, and glutathione reductase (Mkoji et al. 1988a, 1988b). These enzymes inhibit and/or scavenge superoxide ions such as O^-, OH^-, O_2^-, and H_2O_2 produced by the host phagocytic cells (Mauel 1984, Bogdan et al. 1990, Hall and Joiner 1991). For example, inhibition of respiratory burst of host monocytes by *Leishmania* promastigotes was reported by Remaley et al. (1984) and Mc Neeley and Turco (1987). Excretory products from the trematode, *Schistosoma mansoni* were found to be capable of reducing O^-_2 suggesting the presence of antioxidant molecules. The ability to neutralize host oxidative metabolites varies with lifestage of the parasite (Mkoji et al. 1988a, 1988b, Nare et al. 1990). The adults of *S. mansoni* were found to have higher concentrations of antioxidant enzymes than the schistosomula (Mkoji et al. 1988a, 1988b). The tolerance to oxidants by the parasite was positively correlated with

antioxidant concentration of the parasite. The membrane bound acid phosphatase in *L. donavani* is capable of blocking the production of O^-_2, and H_2O_2 by the host neutrophils (Glew et al. 1982).

Acid phosphatases are nonspecific phosphomonoesterase hydrolases which catalyze phosphomonoesters releasing phosphoric acid and corresponding alcohol or phenol (Araki 1993). The optimal pH range of these enzymes is acidic and distinguishes them from similar group of enzymes called alkaline phosphatases. Acid phosphatase hydrolyses phosphate groups from substrates such as B-glycerophosphate (2-glycerophosphate) (Holtzman 1989). The enzyme acid phosphatase in *Leishmania donavani*, a protozoan parasite in humans, was active with phosphomono ester substrates such as fructose 1-6-diphosphate, B-glycerophosphate, glucose-6-phosphate, and glucose-1-phosphate (Gottlieb and Dwyer 1981a, 1981b). Although the physiological role of the acid phosphatase is not known, the membrane bound acid phosphatase in *L. donavani* is believed to cause dephosphorylation of phosphoproteins (Lovelace et al. 1986, Lovelace and Gottlieb 1986) and/or phosphorylated amino acids. Acid phosphatase in *L.donavani* is also beleived to alter the host cell metabolism by dephosphorylation and possibly aid the parasite against host defense mechanisms (Remaley et al. 1985).

Hervio et al (1991) documented the acid phosphatase (AP) activity in *Bonamia ostreae*, an intrahemocytic parasite of the flat oyster *Ostrea edulis* and hypothesized a protective role for acid phosphatase against host defense. *P. marinus* multiplies rapidly within the tissue and cells of the oyster. The *P. marinus* and oyster hemocyte interactions may be similar to the parasite-macrophage interactions known in human and veterinary parasitology for protozoans such as *Leishmania*, *Trypanosoma* or *Toxoplasma*. *P. marinus* posseses high levels of acid phosphatase (0.94 units/mg protein, Volety and Chu, unpublished results) and may be able to evade the host's defense by scavenging the superoxide production by the host. Although how acid phosphatase suppresses superoxide ion production is not known, acid phosphatase may dephosphorylate the enzymes responsible for the production of superoxide ions.

Limited intracellular degradation of phagocytosed trophozoites occurred in oyster hemocytes (La Peyre 1993, Bushek 1994). However, the heavy mortalities of the eastern oysters caused by *P. marinus* at high temperatures and salinities, combined the low degradation rate of *P. marinus* by oyster hemocytes in these studies, suggest that the superoxide dependent killing of *P. marinus* by oyster hemocytes may be ineffective. When trophozoites were used as a stimulant for oyster hemocytes, no chemiluminescence (CL) was observed (La

Peyre 1994, Volety and Chu, unpublished results). However, when zymosan particles were used as a stimulant, the hemocytes elicited CL response. This suggests that certain evasive mechanisms such as suppression or inhibition of superoxide ion production by the host may exist in the trophozoites. Through this mechanism the parasite may escape being killed by superoxide ions produced by the host cell. Thus, it is of interest to know whether *P. marinus* has the ability to suppress the superoxide production by host hemocytes.

Preliminary studies using standard assay procedures have indicated that activities of catalase, glutathione-s-transferase, and superoxide dismutase were not detected in *P. marinus* trophozoites. However, acid phosphatase was detected in both trophozoites and prezoosporangia (Volety and Chu, unpublished results). Our preliminary study indicated that *P. marinus* prezoosporangia contained acid phosphatase activity (0.306 units/10^6 prezoosporangia). Although high temperatures and salinities increase *P. marinus* infection in oysters, it is not known why overwhelming multiplication of the parasite occurs in the host at high temperatures and salinities. Acid phosphatase is also found in oyster serum (Cheng and Rodrick 1975). Whether acid phosphatase in the host serum increases and decreases in the same manner is unknown. An assesment of the levels of acid phosphatase in the host and parasite which would be detrimental to each other needs to be investigated.

The biochemistry of *P. marinus* and its disease processes have not been investigated extensively. It is of interest to know: 1) if *P. marinus* posseses antioxidant enzymes which scavenge the superoxide ions produced by the host hemocytes; 2) if acid phosphatase secretion in *P. marinus* increases with temperature, thus increasing nutrient uptake through dephosphorylation of host organic phosphates and utilizing them for energy and/or reproduction; and 3) if acid phosphatase is invoved in the regulation of cell cycle of *P. marinus*.

Lipid and fatty acid composition of *P. marinus* lifestages:

To establish infection, parasites need to obtain nutrients from the host to grow and multiply. In general, lipids are good sources of energy. Also, fatty acids and phospholipids are essential for membrane synthesis during parasite development and growth. It has been shown that some parasites are capable of secreting lipolytic enzymes to acquire fatty acids and lipids from the host (Szamel and Resch 1981, Vial et al. 1982, Vial et al. 1989, Zidovetzki et al. 1993).

The lipid and fatty acid changes involved in host-parasite relationship have been investigated in molluscs and the helminth parasite, *Echinostoma capri* (Fried et al 1989), reptiles, mammals and the cestode parasite, *Spirometra*

erinacei (Fukushima et al 1988), mammals and cestode parasite, *Hymenolepis diminuta* (Jacobsen and Fairbairn 1967). Few studies have reported the lipid class and fatty acid composition of both the host and the parasite. Fried et al (1989) reported a reduction in the neutral lipids of *E. capri* infected tissues of snail *Biomphalaria glabrata*. Fukushima et. al (1988) observed differences in the fatty acid composition of the plerocercoid and adult stages of the tapeworm *S. erinacei*. Their studies indicated that the proportion of the fatty acid composition in mature and plerocercoid stages closely resembled the host tissue and serum composition, respectively. Biosynthesis and interconversion of fatty acids was demonstrated in the cestode parasite, *H. diminuta* by Jacobsen and Fairbairn (1967). They suggested that the cestode parasite can elongate and assimilate fatty acids of the host through absorption rather than de novo synthesis. Similarly, the malarial parasite, *Plasmodium* spp, is incapable of de novo essential fatty acid and cholesterol synthesis (Sherman 1979, Vial et al. 1984, Zidovetzki and Sherman 1991). However, plasmodia infected erythrocytes contain four to five times more phospholipids than that in uninfected erythrocytes (Holz 1977, Sherman 1979). Most of the new phospholipids are believed to be of plasmodial membrane phospholipid origin. Plasma fatty acids and lysophospholipid were found to serve as sources of the fatty acids required for cellular phospholipid biosynthesis in

the parasite (Holz 1977, Vial et al. 1982, 1984). Due to the absolute requirement of the intracellular parasite for host fatty acids, Vial et al. (1982) has suggested that plasmodial phospholipid metabolism could be a target of chemotherapeutic treatment. To date, nothing is known about the lipid and fatty acid composition of *P. marinus*.

The change from meront to prezoosporangia stage is characterized by appearance of refractile bodies, which are lipid droplets. It is not known if the assimilation of the lipids is from *de novo* synthesis, or accumulation through direct absorption from the host. It is also unknown whether lipid and fatty acids change during parasite development and if temperature and salinity affect the lipid and fatty acid composition. Salinity and temperature are known to affect lipid and fatty acid composition of fish and crustaceans (Sellner and Hazel 1982, Daikoku et al 1982, Chapelle and Zwingelstein 1984). In our preliminary analysis, cholesterol was not detected in trophozoites cultutred in cholesterol deficient medium. Prezoosporangia isolated from infected oyster tissues, however, contained marked levels of cholesterol. This suggests that *P. marinus* may not be capable of synthesizing cholesterol *de novo* and thus is completely dependent on the availability of this compound in host tissues in order to progress into the next developmental stage. Cholesterol is the primary precursor of steroids which are

known to regulate development and growth in marine organisms (Kanazawa et al. 1975, Goad 1976). The role of molt inhibiting hormone (Skinner 1985), the prothoracicotropic hormone, ecdysone, in regulating molting and growth in crustaceans is well established (Smith and Sedlmeier 1990, Chang 1985). Preliminary results also indicate that *P. marinus* have much higher level (> 10% of the total fatty acids) of fatty acid, arachidonic acid (20:4ω6), essential for mammalian and fresh water organisms (Henderson and Tocher 1987). In comparison, the host, oyster tissue has less than 2% of total fatty acids as arachidonic acid. *P. marinus* may be selectively assimilating arachidonic acid and/or modify short chain fatty acids of ω6 from the host. Arachidonic acid is the precursor of many important biochemical components including prostaglandins. Cercaria of *Schistosoma* spp. secrete eicosanoids, which aid the cercaria in penetration of the host tissue by initiating cycloxygenase system. Eicosanoids are metabolites of essential fatty acids. *S. mansoni* produces monopalmitoylphosphatidyl-choline which has detergent like properties. This may aid the parasite in obtaining lipids from the host cell membranes (Furlong 1991) including cholesterol (Golan et al. 1988). Golan et al. (1986) demonstrated that lysophosphatidylcholine from *Schistosoma* spp. lyses human red blood cells. Immune cells which are lysed or are fused with the parasite's surface are not as effective as healthy cells in destroying the parasite

(Golan et al. 1986). Thus, lipids in the parasite may aid the parasite in escaping the hosts' defense. The biochemical charaterization of *P. marinus* at different stages will give us a better understanding of the metabolism of the parasite, and the nutrients required for the development of the parasite within the host. In addition, analysis of the changes in biochemical composition for all the stages of *P. marinus* may enable us to find a clue for interruption of the life cycle of this parasite. Therefore the role of lipids in the parasite development and defense needs to be examined.

The inhibition of specific enzymes which are essential for survival of parasites has been proposed (Ring et al. 1993). Ring et al (1993) generated a computer model to identify novel enzyme inhibitors that play an important role in the life cycle of malaria and schistosome parasites. The larvae of *Schistosoma sps.* secrete a serine protease, a cercarial elastase which aids in penetration of the skin of the human host. Similarly, the malarial parasite, *Plasmodium vivax*, during its erythrocytic phase secretes cysteine protease to degrade hemoglobin. If the synthesis of these enzymes were blocked with specific inhibitors, the cell cycle of the parasite can be disrupted. Similarly, if there is a unique lipid, or fatty acid in a certain cell stage of *P. marinus*, the information can be used for drug development to inhibit and target the compound.

The ability to propagate *P. marinus* in vitro (Kleinschuster and Swink 1993, La Peyre et. al. 1993, Gauthier and Vasta 1993) provides the advantage to investigate the host-parasite interactions. Based on morphology, staining sensitivity by Lugol's stain, and immunoassays using polyclonal antibodies (Dungan and Roberson 1993a), cultured *P. marinus* appear to be identical to *P. marinus* isolated from infected oyster tissue. The minor differences in life cycle still exist between cultured *P. marinus* and those isolated from infected oyster tissue (La Peyre 1993). Future studies should direct efforts to optimize the culture media and conditions. Using cultured cells, I intend to investigate the biochemical aspects of *P. marinus*.

Objectives:

The main objectives of this study is to investigate the transmission and disease processes of *P. marinus* in eastern oysters, *Crassostrea virginica*, including the biochemical aspects of *P. marinus* disease processes. Thus, the specific objective of this study are to investigate:

1) the principal and the most effective lifestage of *P. marinus* disease transmission;

2) synergetic effects of temperature, salinity and dose of infective cells on disease processes of *P. marinus*;

3) the ability of the parasite to suppress/inhibit the reactive oxygen intermediates from host hemocytes;

4) the effects of temperature and salinity on the intra- and extracellular acid phosphatase activity of *P. marinus*;

5) ultrastructural localization of acid phosphatase in *P. marinus*;

6) the lipid and fatty acid composition of the parasite.

CHAPTER 2

COMPARISON OF INFECTIVITY AND PATHOGENICITY OF MERONT (TROPHOZOITE) AND PREZOOSPORANGIAE STAGES OF THE OYSTER PATHOGEN *PERKINSUS MARINUS* IN EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*

ABSTRACT

Two experiments were conducted to compare the infectivity and pathogenicity of two life stages of the parasite *Perkinsus marinus*, meronts (trophozoites) and prezoosporangia (hypnospores), in Eastern oysters, *Crassostrea virginica*. Oysters were inoculated with $5x10^4$ meronts or prezoosporangia/oyster by injection into the shell cavity. Prevalence and intensity of *P. marinus* infection, condition index, serum protein concentrations and lysozyme activities were measured in oysters after 15, 25, 40 and 65 days in Experiment 1 and after 20, 40, 50, 65 and 75 days post-challenge by *P. marinus* cells in Experiment 2. Controls were injected with filtered York River Water. In the first experiment, *P. marinus* infections were initially detected in oysters exposed to prezoosporangia after 15 days post-challenge. In the second experiment, infection was not detected in oysters until 40 days post-challenge with either meronts or prezoosporangia. Intensity and prevalence of *P. marinus* infection were significantly higher ($P < 0.002$) in oysters challenged by meronts compared to prezoosporangia-challenged oysters at the end of both experiments. In Experiment 1, a significant decrease ($P < 0.05$) was observed in serum protein in infected oysters challenged by prezoosporangia compared to uninfected oysters. Condition index was higher in uninfected oysters compared to infected

oysters challenged by prezoosporangia. The differences in condition index and protein were insignificant between oysters infected by meronts or prezoosporangia. Lysozyme activities were significantly lower ($P < 0.05$) in infected oysters than in uninfected oysters challenged with meronts. No significant differences were observed in condition index, protein concentrations and lysozyme activities between oysters challenged by meronts and prezoosporangia in Experiment 2. Lower condition index and protein concentrations in the groups of oysters infected with prezoosporangia compared with the groups infected by meronts and non-challenged at the end of Experiment 1 suggest a higher energetic demand on these oysters.

INTRODUCTION

The once thriving oyster industry in the Chesapeake Bay and east coast of the United States, has been threatened by overfishing, and diseases caused by two protistan parasites, *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo). The effects of the diseases caused by the two protists have been well documented (Andrews 1988, Barber et. al. 1988, Ford 1988, Ford and Figueras 1988, Chu et al 1993, Chu and LaPeyre 1993a, 1993b, Paynter and Burreson 1991). Since 1986, *P. marinus* has reportedly caused greater oyster mortalities in lower Chesapeake Bay than *H. nelsoni* (Andrews 1988).

The life history of *P. marinus* was studied in detail by Perkins (1988). Three lifestages were identified, namely, merozoites, prezoosporangia and the biflagellated zoospores. Immature meronts (merozoites) usually found in the phagosomes of hemocytes are 2-4 µm in size, coccoid, with a fibrogranular wall. As the cells mature, they enlarge to about 10-20 µm with an eccentrically placed vacoule which often contain a refringent vacuoplast. The mature meronts upon repeated karyokinesis and cytokinesis, yield sporangia (schizont, 10-40 µm in size), an 8-32 cell stage enclosed within a mother cell wall (Perkins 1988). Enlargement of meronts to form prezoosporangia is achieved by incubating the meronts in fluid thioglycollate medium (FTM) (Ray 1952). The prezoosporangia

are characterized by extremely large vauole which compresses the cytoplasm into a thin layer against the cell wall. Upon enlargement, the vacuoplast disappears, the nucleus attains a sausage shape, with numerous small lipoid droplets dispersed inside the cell.

Numerous field (Soniat 1985, Craig et al 1989, Soniat and Gauthier 1989, Crosby and Roberts 1990, Gauthier et al 1990, Burreson 1989, 1990) and laboratory studies (Mackin 1951, 1956, 1962, Andrews and Hewatt 1957, Perkins 1966, Chu and LaPeyre 1989, Ragone and Burreson 1993) have investigated the effects of temperature and salinity on the disease processes of *P. marinus* in eastern oysters. Other previous laboratory experiments induced *P. marinus* infection through exposure of oysters to meronts, merozoites and schizonts contained in partially purified or suspension of homogenised infected oyster tissue (Chu and La Peyre 1993a, Hewatt and Andrews 1956, Mackin 1956). For convinience, the cellular stages found in oyster tissue will hereafter be termed meronts with the recognition that merozoites and schizonts are also present.

In nature, the meronts (3-15µm) rarely enlarge to a size of 15-100 µm in moribund oysters and when enlarged are called prezoosporangia (Perkins 1988). Prezoosporangia, when placed in sea water, divide by successive bipartitioning and form

biflagellated zoospores (Perkins 1988, Chu and Greene 1989). Whereas slightly enlarged cells, believed to be prezoosporangia, can be found in moribund oyster, such cells have never been isolated and induced to form zoospores. The presumption is that they have the capability to zoosporulate. Since exposure of oysters to minced oyster tissue containing meronts or freshly isolated and partially purified meronts result in high prevalence of *P. marinus* infection, Perkins (1988) suggested that meronts and merozoites, may be the primary infective agents transmitting disease among oysters in the field with the recognition that zoospores also can induce infections. However, similar infection rates were found by exposing oysters to prezoosporangia and biflagellated zoospores in our laboratory (Chu et al., unpublished results). These results suggest that all the three life stages, namely meronts, prezoosporangia, and biflagellated zoospores are capable of inducing infection in oysters. Some of the previous studies have used minced infected oyster tissue (Hewatt and Andrews, 1956) or minced infected oyster tissue incubated in FTM for one day (Mackin 1962). Therefore, the infective cells used in previous studies would mostly be meronts with some prezoosporangia. None of the previous studies have examined purified prezoosporangia as an infective agent nor were the physiopathological effects investigated. This paper reports the results of experiments in which the infectivity and pathogenicity of meronts and prezoosporangia

were compared. The physiological responses of oysters challenged by these two infective stages were also determined.

MATERIALS AND METHODS

P.marinus diagnosis:

P. marinus infections were diagnosed using hemolymph and tissue assays (Gauthier and Fisher 1990, and Ray 1952). The hemolymph assay was as follows: 300µl of hemolymph containing hemocytes were obtained and incubated in FTM containing antibiotics (penicillin and streptomycin) for 4 days. After incubation, the thioglycollate medium was separated by centrifugation at 800 x g and incubated with 1N NaOH for 1 hour to remove tissue debris and hemocytes. The suspension was then washed twice with water and prezoosporangia stained with Lugol's iodine and counted. Disease intensity was ranked from 1 - 5 (light - heavy). At the end of each experiment, infections were also diagnosed according to the method of Ray (1952) by incubating pieces of rectal and mantle tissue in FTM. Infection intensities were rated as light to heavy (1 - 5) and weighted indices were calculated based on Ray (1954) and Mackin (1962).

Lysozyme activity:

Lysozyme activity was determined spectrophotometrically according to Shugar (1952) and modified by Chu and La Peyre

(1989). Briefly, 0.1 ml of cell-free oyster serum was added to 1.4 ml of bacterial (*Micrococcus lysodiekticus*) suspension. The decrease in absorbance was measured after 1 minute at 450 nm on a Shimadzu UV 600 spectrophotometer. Results are expressed as units/ml of serum. One unit is described as a decrease in absorbance of 0.001 in the bacterial suspension at room temperature in one minute.

Serum protein concentration:

The concentrations of serum protein were measured spectrophotometrically according to Lowry et al. (1951) using bovine albumin as a standard.

Experiments:

Two experiments were conducted to compare the pathogenic effects of meronts and prezoosporangia.

Experiment 1:

Eastern oysters were collected from the Ross's Rock area of the Rappahannock River, Virginia (ambient salinity = 6 ppt, ambient temperature = 19°C). Oysters from this location have the lowest prevalence of *P. marinus* infection of any oyster bed in Virginia (Ragone Calvo and Burreson 1994). Oysters were gradually acclimated over a period of six weeks, to the test conditions (temperature 25.6 ± 1.3°C, salinity 20.7 ± 1.04) in a 200 l tank. Ninety six oysters were then randomly

placed in aerated individual chambers with flowing 1µ filtered York River Water (YRW). Oysters were fed daily during the acclimation and the experimental period with algal paste (0.1 gms/oyster, using a mixture of *Isochrysis galbana*, *Pavlova lutheri*, and Tahitian *Isochrysis galbana*) and water was changed every other day. Meronts were partially purified from infected oyster tissue according to La Peyre and Chu (1994). Prezoosporangia were cultured based on the method described by Chu and Greene (1989). One hundred µl of filtered YRW containing 5 x 10^4 meronts or prezoosporangia cells (meronts cultured in FTM and enlarged to size range of >100µm) were injected into the shell cavity of each oyster. Controls were injected with 1µ filtered YRW. There were three treatments: control, meront challenged and prezoosporangia-challenged oysters. To follow infection development, eight oysters were randomly sampled from each treatment at 15, 25, 40 and 65 days post-challenge. Hemolymph samples were withdrawn from the anterior adductor muscle of individual oysters using a syringe with a 27 gauge needle. Serum lysozyme and protein concentration were measured. Hemolymph was also assayed to evaluate *P. marinus* infection (Gauthier and Fisher 1990). After withdrawal of hemolymph samples, oysters were sacrificed and condition index (CI) (dry meat weight/dry shell weight x 100; Lucas and Beninger 1985) was determined. *P. marinus* infections in oysters were also diagnosed using rectal and mantle tissue according to the tissue assay described by Ray

(1952).

Experiment 2:

The experimental conditions were similar to those of Experiment 1, with the exception that oysters were collected from the Damarsicotta River, Maine, a region beyond the geographical distribution of *P. marinus* (ambient salinity and temperature: 32 - 35 ppt and 12 - 14°C respectively). As in Experiment 1, oysters were gradually adjusted to the test conditions (T = 21.78 ± 0.84°C, S = 20.5 ± 1.19 ppt) in six weeks and then 135 oysters were randomly placed in individual chambers with 1 µm filtered aerated YRW. Nine oysters from each treatment were sampled at the end of 20, 40, 50, 65 and 75 days after being challenged with infective particles. Measurements of CI, serum lysozyme and protein were conducted in individual oysters as indicated above.

STATISTICAL ANALYSES

A one factor analysis of variance (ANOVA) followed by a Tukey-Kramer test was used to determine the differences in CI, lysozyme and protein among treatments. The data were first analyzed for differences among treatments and sampling times. Since some of the oysters were not infected after they were challenged with meronts or prezoosporangia, the CI, lysozyme, and protein data from challenged oysters at all sampling times

from experiment 1, were split into infected and uninfected oysters. Data from uninfected oysters from each treatment at all sampling times were pooled with the controls. This resulted in three groups, namely, uninfected, meront-infected and prezoosporangia-infected. Data were then reanalyzed using One-Way ANOVA to determine differences among groups. In Experiment 2, CI, lysozyme and protein data were analyzed using One-Way ANOVA without splitting into infected and uninfected groups. Logistic regression (Agresti 1990) was used to determine differences in prevalence of infection between treatments and sampling times in both the experiments.

RESULTS

In Experiment 1, infection first appeared in oysters 15 days after being challenged with prezoosporangia and 25 days after challenge with meronts (Fig 1a). Prevalence, at 65 days post challenge was higher in oysters challenged by meronts (87.5%), compared to oysters challenged by prezoosporangia (43%) (Fig 1a). Prevalences of both groups significantly increased with time ($p < 0.05$). Prevalence was not significantly different between meront-challenged and prezoosporangia-challenged oysters. Intensities of infections ranged from light to heavy (1 - 5) in meront-challenged oysters, whereas no heavy infections were detected in prezoosporangia -challenged oysters (Fig 2a). When intensity

of infection was expressed as weighted incidence (sum of disease code number/ total number of oysters examined), it showed a trend similar to that of prevalence. Weighted incidence (Table 1) at the end of the experiment was higher in oysters challenged with meronts (2.13), compared to oysters challenged with prezoosporangia (0.86).

In Experiment 2, the first infections appeared after 40 days in both meront and prezoosporangia-challenged oysters. Prevalence (Fig 1b) was significantly ($p < 0.002$) higher in meront-challenged oysters (77.5%) compared to prezoosporangia challenged oysters (57.2%). As in Experiment 1, infection in both groups increased with time ($p < 0.0001$). Intensities of infections ranged from light to moderate heavy (1 - 4) in oysters challenged with meronts whereas only light infections (1) were observed in prezoosporangia challenged oysters (Fig 2b). Weighted incidence (Table 1) at the end of the experiment was higher in meront challenged oysters (0.86) as compared to prezoosporangia challenged oysters (0.5).

There were no differences in CI, lysozyme and protein concentrations among treatments at different sampling times in Experiment 1 ($p > 0.05$). In Experiment 1, within the prezoosporangia-challenged group, CI of infected oysters were lower than uninfected oysters (Fig 3). The CI of infected

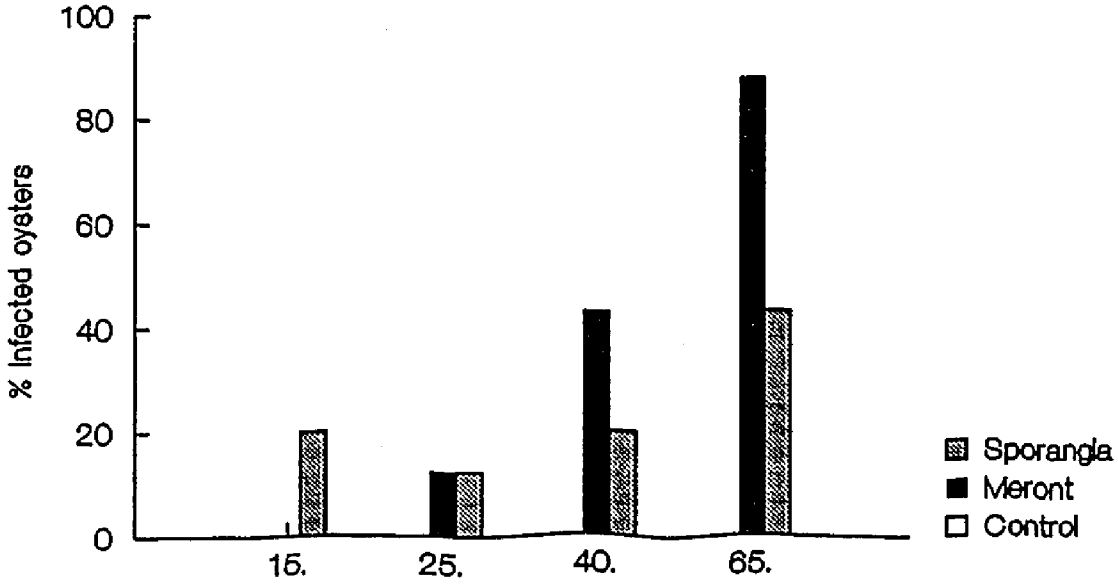
oysters from the group challenged by meronts was not different from infected oysters from the group challenged by prezoosporangia ($p > 0.05$). Serum protein concentrations in infected oysters challenged with prezoosporangia were significantly lower ($p < 0.05$) than the uninfected oysters (Fig 4). However, no significant difference in proetin concentrations were observed between infected and uninfected oysters in the group of oysters challenged with meronts. No differences ($p > 0.05$) were observed in serum protein concentrations between meront and prezoosporangia challenged oysters. Also, no significant difference in protein concentrations was observed between infected oysters challenged with meronts or prezoosporangia. In oysters challenged by meronts, lysozyme activity was significantly higher ($p < 0.05$) in uninfected than infected oysters (Fig 5). No such differences were observed between infected and uninfected oysters challenged with prezoosporangia.

In Experiment 2, CI and serum protein concentrations significantly decreased ($p < 0.05$) in all treatments with time. The CI of oysters at the end of 20 days was significantly higher than the CI of the oysters at the end of 50 and 75 days (Fig 6). Protein concentrations in oysters from all treatments decreased with time (Fig 7). Protein concentrations at the end of 20, 40 and 50 days were significantly ($p < 0.05$) higher than at the end of 65 and 75

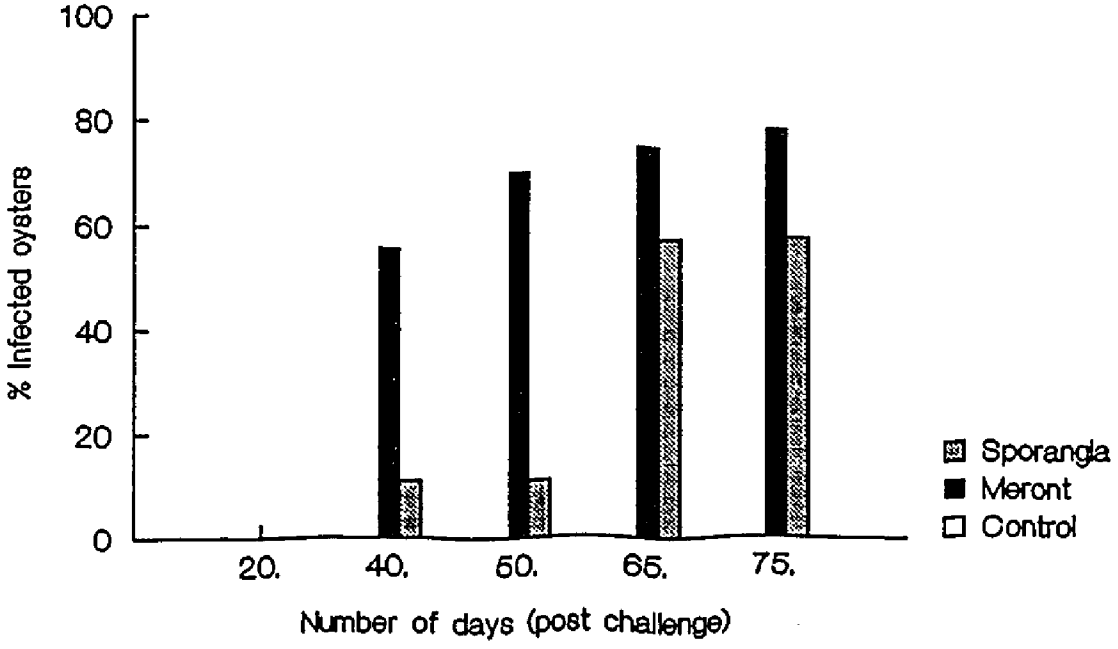
days post-challenge (Fig 7). No significant differences were observed in lysozyme activities between treatments at any sampling time.

Figs 1a and 1b: *P. marinus* prevalence in oysters after 15, 25, 40, and 65 days postchallenge (Fig 1a) and 20, 40, 50, 65, and 75 days postchallenge (Fig 1b) by meronts or prezoosporangia.

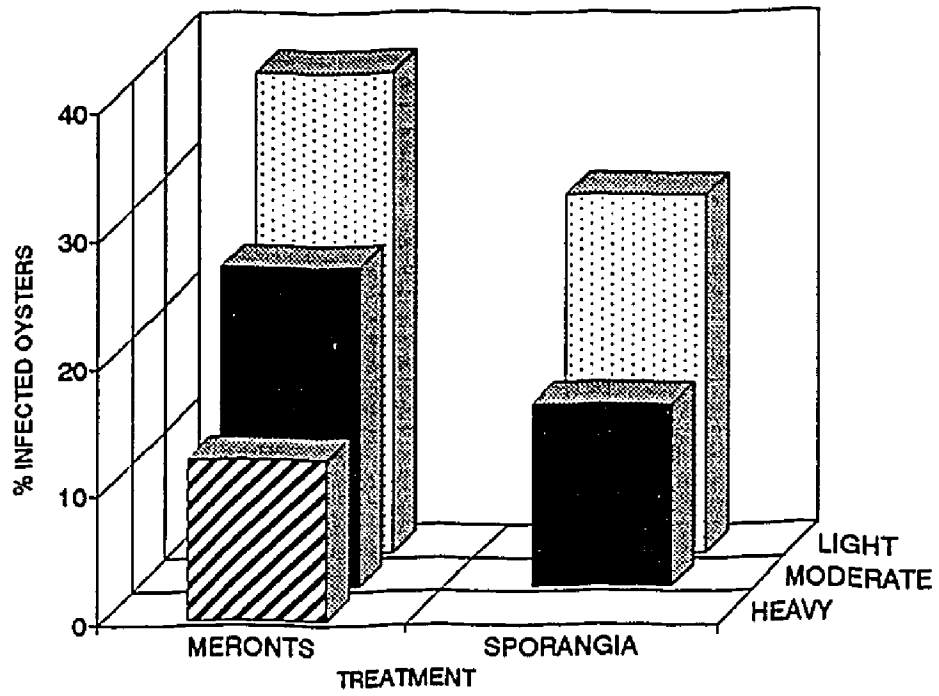
Trial 1



Trial 2



Figs 2a and 2b: *P. marinus* infection intensity in oysters from experiment 1 (Fig 2a) after 65 days and experiment 2 (Fig 2b) after 75 days postchallenge by meronts and prezoosporangia.


40
30
20
10
0
% INFECTED OYSTERS
LIGHT
MODERATE
HEAVY
MERONTS
SPORANGIA
TREATMENT

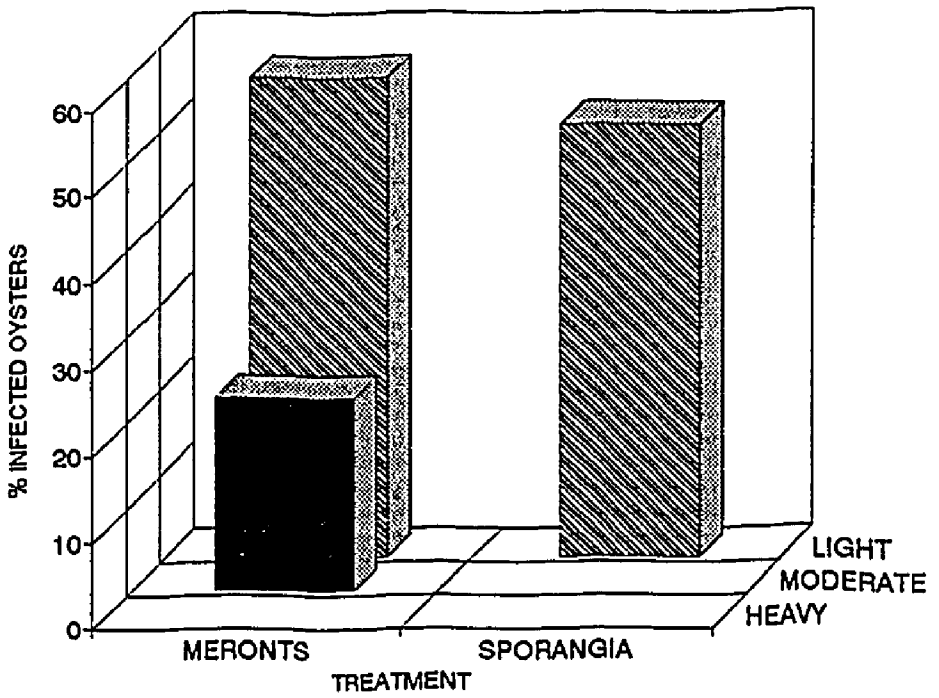

60
50
40
30
20
10
0
% INFECTED OYSTERS
LIGHT
MODERATE
HEAVY
MERONTS
SPORANGIA
TREATMENT

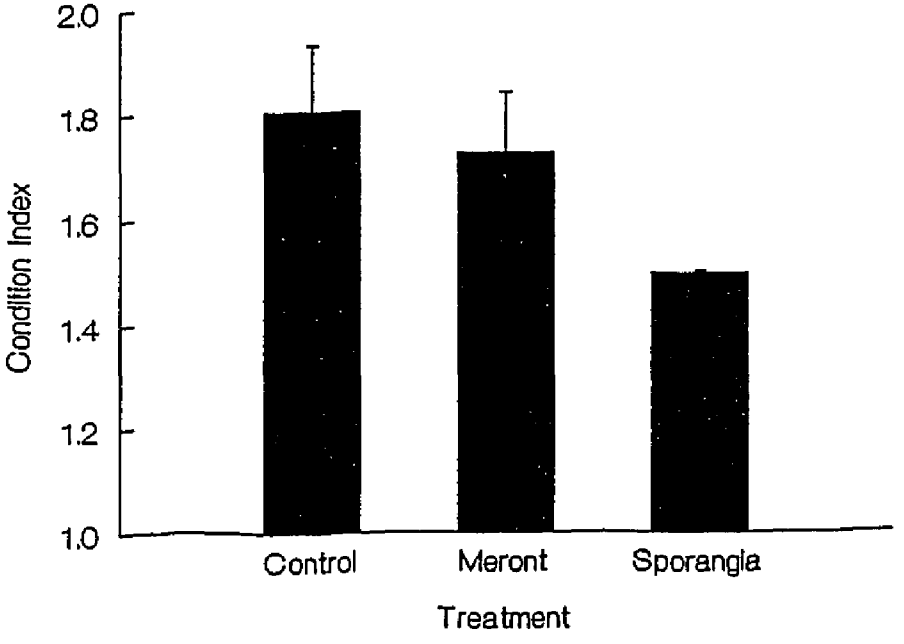
Table 1: Weighted incidence of *P. marinus* infection and experimental conditions

TABLE 1:

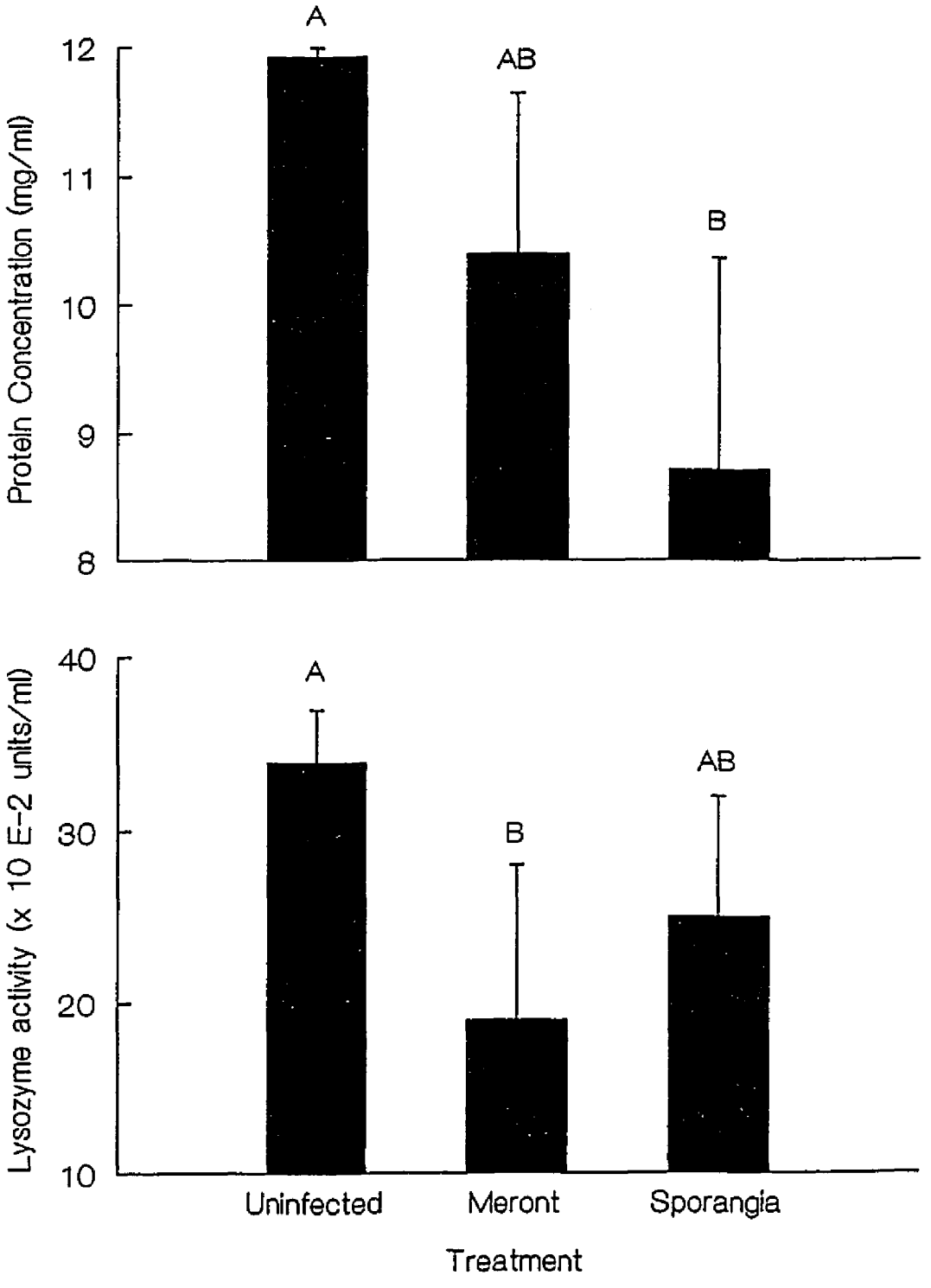
P.marinus INFECTION:

INFECTIVE CELL	WEIGHTED INCIDENCE		EXPERIMENTAL CONDITIONS	
	EXPT1	EXPT2	EXPT1	EXPT2
MERONT	2.13	1.33	T=25.6±1.33°C	T=21.78±0.84°C
PREZOOSPORANGIA	0.86	0.5	S=20.7±1.04PPT	S=20.5±1.19PPT

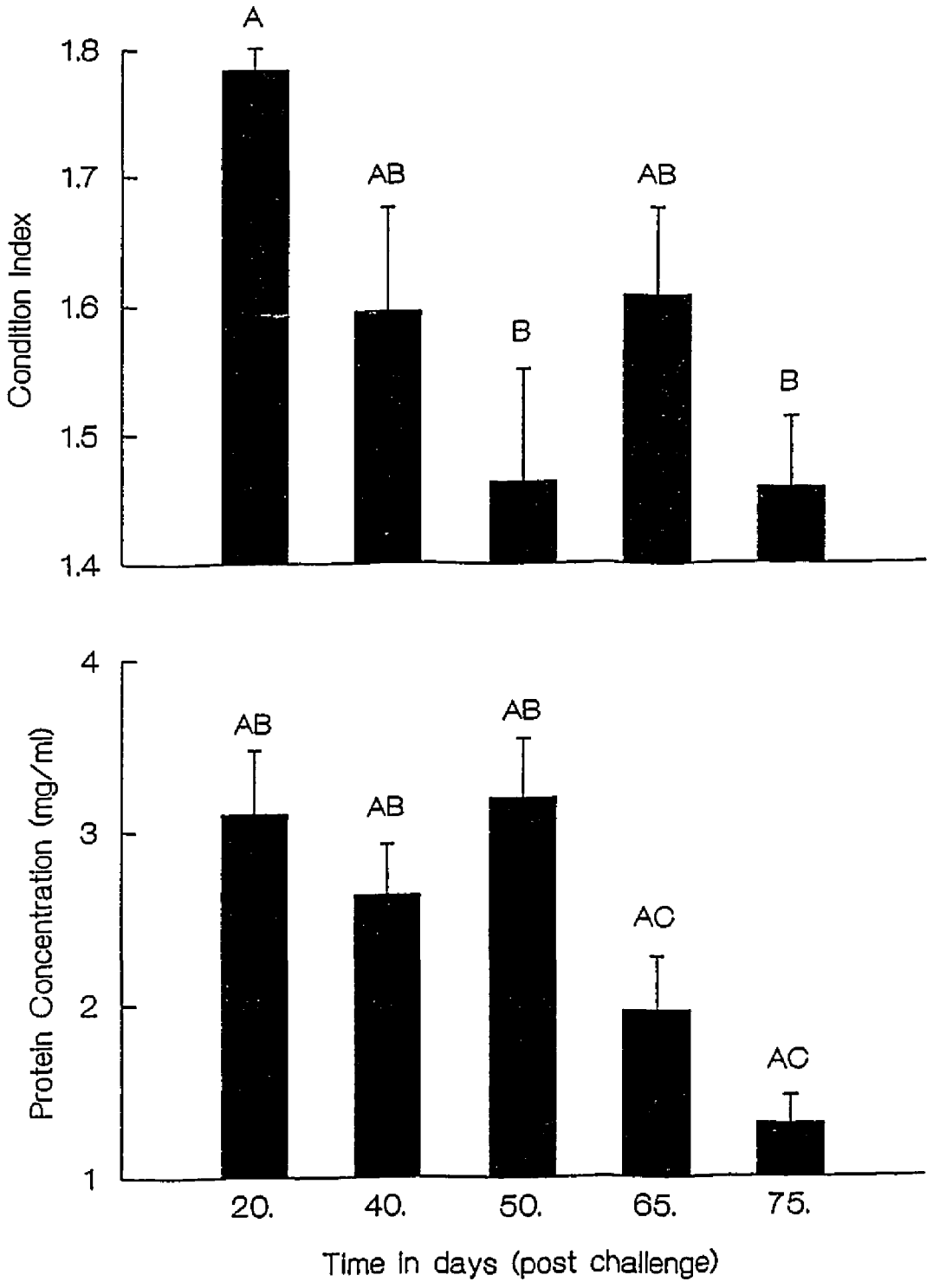
Fig 3: Mean CI (± SE) in uninfected, meront- and prezoosporangia-challenged oysters.


2.0
1.8
1.6
1.4
1.2
1.0
Condition Index
Control
Meront
Sporangia
Treatment

Figs 4 and 5: Mean serum protein concentration (±SE) (Fig. 4) and mean serum lysozyme activity (±SE) (Fig. 5) uninfected and infected oysters challenged by meront and prezoosporangia. Bars with similar letters are not significantly different ($p < 0.05$).


A
AB
B
12
11
10
9
8
Protein Concentration (mg/ml)
A
AB
B
40
30
20
10
Lysozyme activity (x 10 E-2 units/ml)
Uninfected
Meront
Sporangia
Treatment

Figs 6 and 7: Mean CI (± SE) (Fig 6) and serum protein concentration (±SE) (Fig 7) in oysters at the end of 20, 40, 50, 65 and 75 days post-challenge. Bars with similar letters are not significantly different ($p > 0.05$)


A
AB
AB
B
B
1.8
1.7
1.6
1.5
1.4
Condition Index
AB
AB
AB
AC
AC
4
3
2
1
Protein Concentration (mg/ml)
20.
40.
50.
65.
75.
Time in days (post challenge)

DISCUSSION

Results of the present study show that both meronts and prezoosporangia infect oysters with meronts being more infective than prezoosporangia. This supports the hypothesis (Perkins 1988) that meronts are the primary agents of disease transmission of *P. marinus* in oysters. The higher prevalence of infection in oysters challenged with meronts might have been due to the higher virulence of meronts. The meronts may multiply rapidly in oysters at warm temperatures, such as those (Table 1) used in the present study. The cause for the lower infection rate of prezoosporangia is not clear. Although the prezoosporangia injected into the oysters were > 95% viable at the time of infection, viability may drop after injection into the oyster tissue, resulting in lower infections. The prezoosporangia used in this study have been cultured in FTM which may have affected their infectivity. In the field, the infectivity of prezoosporangia could be different. Oysters challenged with cells from pure cultures of *P. marinus* (meronts, merozoites and schizonts) did not exhibit as heavy infections, as those obtained using meronts in homogenized oyster tissue (Volety and Chu, unpublished results, Bushek et al. 1993). Culture of *P. marinus* in artificial media may reduce virulity of the cell stages.

Division of prezoosporangia into meront-like structures

by schizogony has been observed in culture (La Peyre 1993; Perkins, personal communication). Although sporangia divide and release biflagellated-zoospores in sea water (Perkins 1976, Chu and Greene 1989), the production of zoospores by meronts or prezoosporangia in oyster tissue, or in cells isolated from oyster tissue without FTM treatment has not been documented. Indeed, the production of biflagellated-zoospores and their subsequent release into sea water may not take place in oyster tissue. Furthermore, the fate of inoculated prezoosporangia in oyster tissue is not known. The lower prevalence in oysters challenged with prezoosporangia may be the result of a long lag time in the division of sporangia into meronts, and/or the high mortality rate of cells induced to form prezoosporangia.

Dittman (1993) reported insignificant differences in CI between lightly infected and uninfected oysters. However, in the same study, significantly lower CI values were observed in heavily infected oysters compared to uninfected ones. Lower CI in infected oysters challenged by prezoosporangia compared to uninfected oysters in Experiment 1, though not statistically significant (Fig 3), may be because, only a few of the oysters were heavily infected. The decrease in condition index of oysters with time in Experiment 2, may be due to the stress in the confined environment.

The results from Experiment 1 indicated that infected oysters challenged by prezoosporangia had significantly lower protein concentrations than uninfected oysters. Lower tissue and hemolymph protein has been observed in oysters heavily infected by *Haplosporidium nelsoni* (Ford 1986a, 1986b, Barber et al 1988, Ling 1990). However, no significant differences in protein concentrations were observed in oysters lightly infected by *P. marinus* as compared to uninfected oysters (Chu and La Peyre 1993a).

Lysosomal enzymes are believed to play a role in defense in both vertebrates and invertebrates (Ingram 1980, Jolles and Jolles 1984), including molluscs (Mc Dade and Tripp 1967a, b, Cheng 1981, 1983, Huffman and Tripp 1982, Moore and Gelder 1985, Chu 1988). Lysozyme activity in oysters was observed to be negatively correlated with *P. marinus* infection and temperature (Chu and LaPeyre 1993a). Lysozyme activities of uninfected oysters in Experiment 1 had significantly higher activities than infected oysters challenged with meronts (Fig 5). Lysozyme is hypothesized to be an important enzyme in resistance to *P. marinus* infection (Chu et al. 1993). The absence of *P. marinus* infection in some of the oysters may have been as a result of higher serum lysozyme activity which may explain the significantly higher lysozyme activity in uninfected oysters. However, no difference in lysozyme activity was observed between meront challenged and

prezoosporangia challenged oysters.

The higher prevalence, intensity and weighted indices of *P. marinus* infections in Experiment 1 compared to Experiment 2 may be due to the higher temperature in the former experiment (Table 1). Temperature is one of the two most important factors (the other being salinity) influencing the geographic distribution of *P. marinus* in oysters. Chu and La Peyre (1993a) reported that prevalence and intensity of *P. marinus* infection increased with increasing temperature. In their study the prevalence of *P. marinus* in oysters was 23, 46, 91 and 100% at 10, 15, 20 and 25°C respectively. *P.marinus* infection is also positively correlated with temperature in the field (Soniat 1985, Criag et al 1989, Soniat and Gauthier 1989, Crosby and Roberts 1990, Gauthier et al. 1990). The batches of *P. marinus* meronts used for the challenging the oysters in the two experiments were isolated from different infected oysters. Their relative infectivity and virulence could differ contributing to the different infection rates. The difference in the source of oysters may also have been one of the factors for lower incidence of *P. marinus* infection. Differences in susceptibility of oysters from different populations to *P. marinus* infection have been reported (Chu and La Peyre 1993b, La Peyre 1993). Their studies have shown differences in prevalence of *P. marinus* infection in oysters from three locations in Chesapeake Bay

and between Chesapeake Bay and Gulf oysters. Habitat and genetic dissimilarities were suggested as the reasons for the differences in prevalence of infection.

Since only light infections were detected in Experiment 2 in both oysters challenged with meronts and prezoosporangia, the insignificant differences noted in condition index, lysozyme activity, and protein concentrations between different treatments were not surprising. These results agree with the findings by Dittman (1993), and Chu and La Peyre (1993a). Neither found differences in CI, lysozyme and protein concentrations between lightly infected and uninfected oysters.

In summary, meronts are more infective than prezoosporangia and are possibly the principal agents of disease transmission in the field. The lower condition index and protein values in the treatment of infected oysters challenged with prezoosporangia, compared with uninfected and meront challenged oysters, suggest that prezoosporangia may be exerting a higher energetic demand on the host than do meronts. Further studies are needed to examine the causes for the lower protein concentrations in prezoosporangia-challenged oysters.

CHAPTER 3

DISEASE PROCESSES OF THE OYSTER PATHOGEN, *PERKINSUS MARINUS* IN THE AMERICAN OYSTER, *CRASSOSTREA VIRGINICA*: SYNERGETIC EFFECTS OF PATHOGEN DOSE, TEMPERATURE AND SALINITY.

ABSTRACT

Experiments were conducted (i) to test the response of oysters, *Crassostrea virginica*, to different doses of two life stages of the oyster pathogen, *Perkinsus marinus*; and (2) to determine if any synergistic effect exists between temperature, salinity and dose of *P. marinus* cells on the infection prevalence and intensity in oysters. To test the response of oysters to different doses of *P. marinus* meronts, 10, 10^2, 10^4, or 10^5 meronts or prezoosporangia were inoculated into the shell cavity of oysters. Synergistic effects of temperature, salinity, and dose of *P. marinus* cells were determined by challenging oysters with two different doses (2.5×10^3 or 2.5×10^4 meronts/oyster) of meronts at nine temperature-salinty combinations using 10, 15, and 25°C and 3, 10 and 10ppt. Condition index, total number of hemocytes and percentage of granulocytes, serum protein and lysozyme concentrations were also measured. Results indicate that increased infection prevalence and intensity occurred at high temperatures and salinities and there was a dose dependent response to infective particles. The minimum number of *P. marinus* cells required to initiate infection in oysters was $10\text{-}10^2$. Temperature was the most important factor, followed by the dose of infective particles, influencing the susceptibility to *P. marinus* and subsequent disease development in oysters. Salinity was less important a factor

than temperature and meront doses. The effect of interactions among these three factors on disease prevalence was not significant. However, the interaction between temperature and salinity; and between temperature and dose significantly influenced the infection intensity. Temperature and salinity significantly affected the cellular and humoral factors in oysters.

INTRODUCTION

Diseases caused by the two protistan parasites, *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo), in combination with overfishing, have threatened the oyster industry in the Chesapeake Bay and east coast of the United States. Severe oyster mortalities during the 1940s in the Gulf of Mexico led to the discovery of *P. marinus* by Mackin et al. (1950). Since then, numerous studies have been conducted to investigate the susceptibility, infectivity and transmission of this parasite, including the pathogenic effects on the host organism (Andrews 1988, Chu et al 1993, Chu and LaPeyre 1993a, 1993b, Paynter and Burreson 1991, Volety and Chu 1994).

The subtropical distribution of *P. marinus* has led to the hypothesis that temperature is one of the important factors influencing infection in oysters. Temperature affects the growth rates of *P. marinus* in vitro (Chapter 4) and metabolic rate of the parasite (Burreson et al. 1994). On the other hand, *P. marinus* infection was more abundant and intensified in areas of high salinity, indicating that salinity is another limiting factor for *P. marinus* infection in oysters. Scott et. al (1985) suggested that the physiological changes in the oyster due to changes in salinity contribute to the altered susceptibility of oysters to *P. marinus* and their later mortality. Numerous field (Soniat 1985, Craig et al 1989,

Soniat and Gauthier 1989, Crosby and Roberts 1990, Gauthier et al 1990, Burreson 1989, 1990) and laboratory studies (Mackin 1951, 1956, 1962, Andrews and Hewatt 1957, Perkins 1966, Chu and LaPeyre 1989, Ragone and Burreson 1993, 1994, Fisher et al 1992) have revealed a positive correlation between *P. marinus* infection, temperature, and salinity. Dosage of infective elements has also been considered an important factor in the disease prevalence in oysters. Mackin (1956) suggested that the low *P. marinus* infection in areas of low salinity is due to the dilution of infective elements by inflow of fresh water, rather than the effect of salinity on the parasite or host. Mackin (1956) investigated the dose responses of oysters to various *P. marinus* infective cells (10 - 10^6) in minced oyster tissues, previously incubated in thioglycollate medium for 24 hours, and found that the mortality caused by the disease in oysters is a function of the "dose" of infective cells. Mackin's (1956) study only monitored the oyster mortality caused by exposure to *P. marinus* cells, but not the infection rate, intensity, or the physiopathologic effects in oysters.

All the three life stages of *P. marinus* (namely, meronts, prezoosporangia and zoospores) are capable of inducing Dermo infection in oysters (Chu et al., unpublished results, Perkins 1988). Recent studies (Volety and Chu 1994, Perkins 1988) suggested that meronts are the principal causative elements

and the most effective in initiating Dermo infection in oysters. However, the minimum dose of *P. marinus* required for initiating infection in oysters, and the results of the interaction of temperature, salinity, and dose of *P. marinus* cells on Dermo infection in oysters have not been examined. This paper reports results of experiments conducted to: (i) investigate the dose response and the minimum dose of meront and prezoosporangiae life stages required to initiate *P. marinus* infection in oysters; and (ii) determine the interactive effects of temperature, salinity and different doses of *P. marinus* meronts on the response of oysters.

MATERIALS AND METHODS

Preparation of meront suspension

Meront suspension was prepared as follows: *P. marinus* infected oyster tissues were rinsed thoroughly with filtered (0.22 µm) York River water (YRW) and subsequently homogenized in 0.22 µm filtered YRW with a blender (Virtis, Model 23) at high speed for 2 minutes. The suspension was then passed through a series of 100, 50, 35 and 20 µm meshes to remove oyster tissue residues (La Peyre and Chu, 1994). Meronts contained in the filtrate were further purified by repeated centrifugation and washing. The number of meronts in suspension was counted using a hemacytometer and adjusted to the desired concentration, as described in the experiments below.

Preparation of hemocytes and sera

Hemocytes and sera were prepared as follows: hemolymph was withdrawn from the adductor muscle sinus with a syringe through notches in the shell and hemolymph was placed in test tubes on an ice bath. Total hemocyte number and percent of granulocytes were counted in individual hemolymph samples (N=5) using a hemacytometer. For humoral activity measurements, serum of each hemolymph sample was separated from hemocytes through centrifugation (400 x g at 4°C for 10 min). Sera were withdrawn and stored at -20°C for other analyses.

P. marinus diagnosis

P. marinus infections were diagnosed using hemolymph and tissue assays (Gauthier and Fisher 1990, Ray 1952). The hemolymph assay was as follows: 300µl of hemolymph containing hemocytes were obtained and incubated in fluid thioglycollate medium (FTM) containing antibiotics (penicillin and streptomycin) for 4 days. After incubation, the thioglycollate medium was separated by centrifugation at 800 x g and incubated with 1N NaOH for 1 hour to remove tissue debris and hemocytes. The pellet (prezoosporangia) was washed twice with water and stained with Lugol's iodine. The number of prezoosporangia was counted under an inverted microscope (Nikon). Disease intensity was ranked 0, 1, 3, 5 (negative, light, moderate, and heavy) based on the number of

prezoosporangia present in the hemolymph sample. At the end of each experiment, infections were also diagnosed according to the method of Ray (1952) by incubating a piece of rectal and mantle tissue in FTM. Weighted indices (weighted prevalence, sum of disease code number/number of oysters) were calculated according to Ray (1954) and Mackin (1962).

Lysozyme activity

Lysozyme activity was determined spectrophotometrically according to Shugar (1952) and modified by Chu and LaPeyre (1989). Briefly, 0.1 ml of cell-free oyster serum was added to 1.4 ml of bacterial (*Micrococcus lysodiekticus*) suspension (Sigma, USA). The decrease in absorbance due to the lysis of bacterial cell wall was measured at 450 nm on a Shimadzu UV 600 spectrophotometer after 1 minute. Results are expressed as concentration (µg lysozyme/ml of serum). Hen egg white lysozyme dissolved in appropriate salinity water (3, 10 or 20 ppt) was used in constructing the standard curve.

Serum protein concentration

The concentrations of serum protein were assayed according to Lowry et al. (1951) using bovine serum albumin as a standard. Ten µl of a cell-free hemolymph sample from individual oysters were used for the serum protein measurement.

Experiments:

EXPERIMENT 1:

Response of oysters to different doses of meronts and prezoosporangia:

Two trials were conducted in this study. Oysters used in both trials were obtained from Damarsicotta River, Maine, a region beyond the geographical distribution of *P. marinus*. The ambient temperature and salinity at the time of collection were T=15°C and S=30 ppt and 20°C and 30 ppt, respectively in Trials 1 and 2. The oysters were gradually acclimated over a period of six weeks to the test temperature and salinity of 25°C and 14 ppt in Trial 1 and to 22°C and 21 ppt in Trial 2. A total of 150 oysters (5 - 6.5 cms size range) were collected from Damarscicotta River for each trial. Hemolymph was withdrawn from 15 randomly selected oysters using a syringe with a 27 gauge needle. Total hemocyte number (THC) and percentage of granulocytes (PG) were counted using a hemacytometer. After hemolymph withdrawal, pieces of rectal, mantle and digestive gland from individual oysters were excised and used for *P. marinus* infection diagnosis according to Ray (1952). All the oysters were diagnosed to be free of *P. marinus* infection at that time. Condition index (CI) (CI = dry meat weight/dry shell weight x 100; Lucas and Beninger 1985)) of the oysters were then determined according to Lucas and Beninger (1985). The remaining 135 oysters were divided

into nine groups (groups challenged with 10, 10^2, 10^4, or 10^5 meronts or prezoosporangia and a control group, 15 oysters per group) and oysters were randomly arranged in individual plastic chambers with 1µ filtered aerated YRW. Water was changed every other day and the oysters fed daily with 0.2 gms/oyster algal paste (*Thalassiosira weissflogii*). *P. marinus* meronts or prezoosporangia in 100 µl of 1µm filtered YRW containing 10, 10^2, 10^4, or 10^6 were injected into the shell cavity of individual oysters. Oysters from the control group received only filtered YRW. Oysters were sampled for hemolymph after 12 and 8 weeks post-challenge by *P. marinus*, for Trials 1 and 2 respectively and CI was then determined. *P. marinus* infection in oysters was also determined using the tissue assay (Ray 1952).

EXPERIMENT 2:

Synergetic effects of temperature, salinity and dose of *P. marinus* meronts in oysters:

Five hundred oysters were collected from Damarsicotta River, Maine (ambient temperature 0°C, salinity 32 ppt). All the oysters were gradually acclimated (acclimation time = eight weeks) in 1000 l tanks with 1 µm filtered water at nine combinations of test temperatures and salinities: 3 ppt at 10, 15 and 25°C; 10 ppt at 10, 15 and 25°C; and 20 ppt at 10, 15 and 25°C. Oysters were fed 0.1 gm of algal paste/oyster/day. Before the commencement of the experiment, 30 randomly

selected oysters were sacrificed and each oyster examined for condition index and *P. marinus* infection. After the oysters were acclimated to the respective test temperatures and salinities, they were divided into nine groups of different test temperatures and salinities and randomly placed in individual chambers with 1 µm filtered aerated YRW, and adjusted to test salinities and temperatures. *P. marinus* meronts were isolated from infected oyster tissue and adjusted to a concentration of 2.5×10^4 or 2.5×10^5 cells/ml in YRW. One hundred µl of YRW containing 0, 2.5×10^3 or 10^4 meronts were injected into the shell cavity of individual oysters (N=15). Controls were injected 100 µl of filtered YRW. Hemolymph was withdrawn from oysters after eight weeks postchallenge with *P. marinus* to determine THC and PG counts, protein and lysozyme concentrations. Oysters were then sacrificed and examined for *P. marinus* infection and CI. Infection intensities and weighted indicies were ranked according to Mackin (1962). Mortalities of oysters during the experimental period were recorded and examined for *P. marinus* infection.

STATISTICAL ANALYSES

Logistic regression and log linear modelling (Agresti 1990) were used to determine: (i) differences in prevalence of infection in oysters inoculated different doses of either

meronts or sporangia in both the dose experiments; (ii) the significance of the effects of temperature, salinity and doses of *P. marinus* infective cells and their interaction on *P. marinus* susceptibility of oysters in the synergetic effects experiment. To determine the differences in CI of oysters due to *P. marinus* cell stage and dose of each cell stage, a two-way ANOVA was used in the dose response experiment (Experiment 1), while a three way ANOVA was used to determine the differences in THC, PG, CI, protein, and lysozyme in oysters in synergetic effects experiment (Experiment 2). A multiple comparison test (TUKEY) was used to determine the differences among treatment means. CI data in Experiment 1 were SIN transformed.

RESULTS

1) Response of oysters to different doses of *P. marinus* meronts and prezoosporangia:

Prevalence and intensity of *P. marinus* infection.

A dose dependent response of *P. marinus* infection was observed in oysters. *P. marinus* infection in oysters increased ($p < 0.0001$) with increasing dose of *P. marinus* cells (Figs 1a and 1b). The prevalence of infection in oysters challenged with 10, 10^2, 10^4, and 10^5 meronts was 0, 13, 50 and 71% respectively in Trial 1 while the prevalence was 0, 13, 67, and 80% respectively in Trial 2. Meront-

challenged oysters had a significantly higher ($p < 0.05$) infection rate compared to prezoosporangia-challenged oysters (Figs 1a and 1b). Oysters challenged with prezoosporangia had a prevalence of 0, 14, 20 and 33% in trial 1 and 0, 7, 29 and 53% in trial 2 at the same concentration of meronts indicated above. No *P. marinus* infection was detected in control oysters. Results indicate that the minimum dose of *P. marinus* cells required to initiate infection in oysters is between 10 and 10^2 cells/oyster (Figs 1a and 1b).

Results of weighted incidence of *P. marinus* infection were similar to that of prevalence of infection. Intensity of *P. marinus* infection in oysters also increased with increasing number of *P. marinus* cells inoculated into the oyster shell cavity. The infection intensity in oysters challenged with meronts was generally higher, compared to those challenged with prezoosporangia (Fig 2a and 2b).

THC, PG, CI:

No significant differences were observed in THC and PG of oysters challenged by either meronts or prezoosporangia and between challenged doses ($p > 0.05$) in either trials. Significant differences were found in CI of oysters challenged with prezoosporangia versus meronts in Trial 1. Prezoosporangia-challenged oysters had a significantly lower CI compared to meront-challenged oysters ($p < 0.05$) (Fig 3).

However, no significant differences in CI were observed between meront-challenged and prezoosporangia-challenged oysters in Trial 2 ($p > 0.05$).

2) Synergetic effects of temperature, salinity and doses of *P. marinus* meronts on responses of oysters:

Mortality:

Heavy mortalities occurred in oysters at 3 ppt during the acclimation period. Mortalities were also heavy in oysters maintained at 25°C (34 out of 45) compared to 15°C (12 out of 45) and 10°C (10 out of 45). Only 3 oysters at 10 ppt and 2 oysters at 20 ppt died at all temperatures. However, none of the oysters were found to be infected with *P. marinus*. Since all the oysters which were dead, were before inoculation, they were eliminated from prevalence calculations.

Prevalence and intensity of *P. marinus* infection.

P. marinus prevalence in oysters increased with increasing temperature ($p < 0.0001$) and salinity ($p < 0.0003$), and there was a dose dependent response to *P. marinus* meronts ($p < 0.0001$) (Fig 4). Based on the results of statistical analyses, temperature is the most important factor followed by dose of infective particles in influencing *P. marinus* susceptibility and subsequent disease development in oysters. Salinity was less important than temperature and meront doses.

Interaction between the three factors on disease prevalence was not significant.

Intensity of *P. marinus* infection in oysters increased with the increasing temperature ($p < 0.0001$), salinity ($p < 0.01$) and meront dose ($p < 0.0001$) (Figs 5a, 5b, and 5c). There was a significant interaction effect between temperature and salinity (Fig 6a) ($p < 0.0001$); and between temperature and meront dose (Fig 6b) ($p < 0.01$) on intensity of *P. marinus* infection. However, the effect of interaction between salinity and meront dose was insignificant. Similar to prevalence, the interaction of the three factors, temperature, salinity and dose of *P. marinus* meronts on the intensity of infection in oysters was not significant.

CI, THC, PG, protein and lysozyme concentrations.

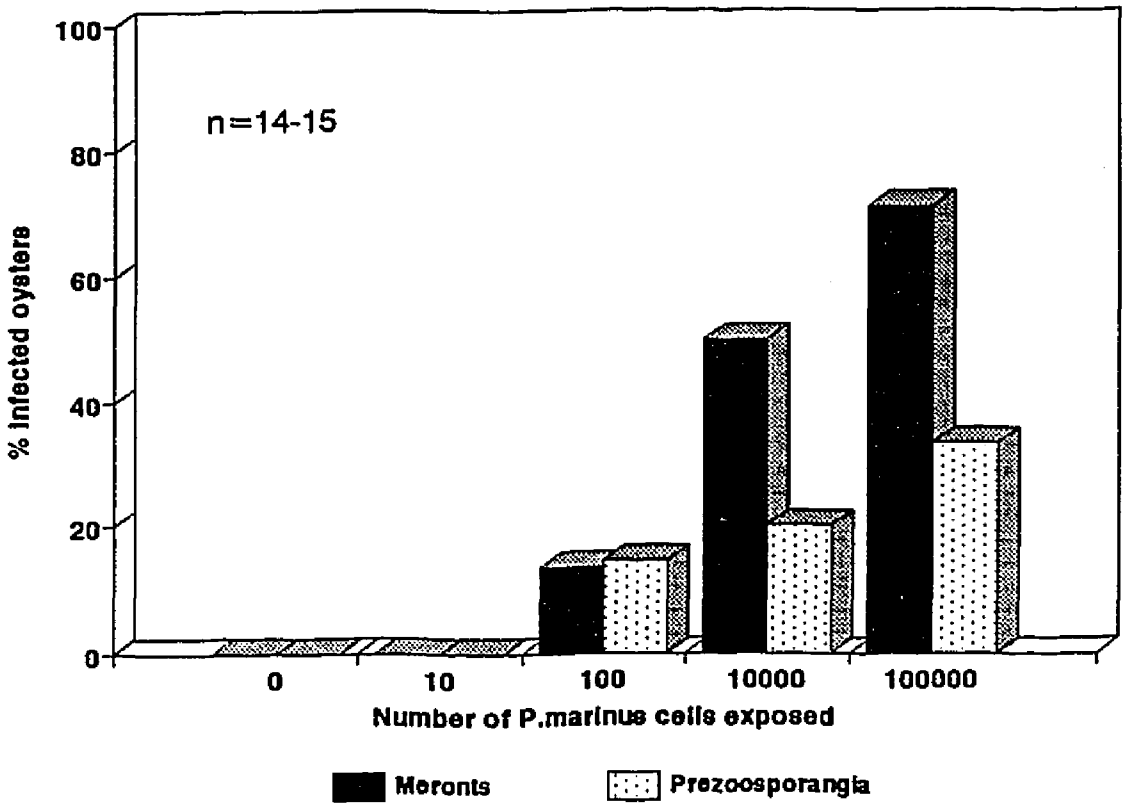
CI of oysters was significantly reduced with increasing temperature ($p < 0.0001$) (Fig 7). Salinity and dose of meronts did not significantly affect the CI of oysters. CI of infected oysters was significantly lower compared to uninfected oysters ($p < 0.05$) (Fig 8).

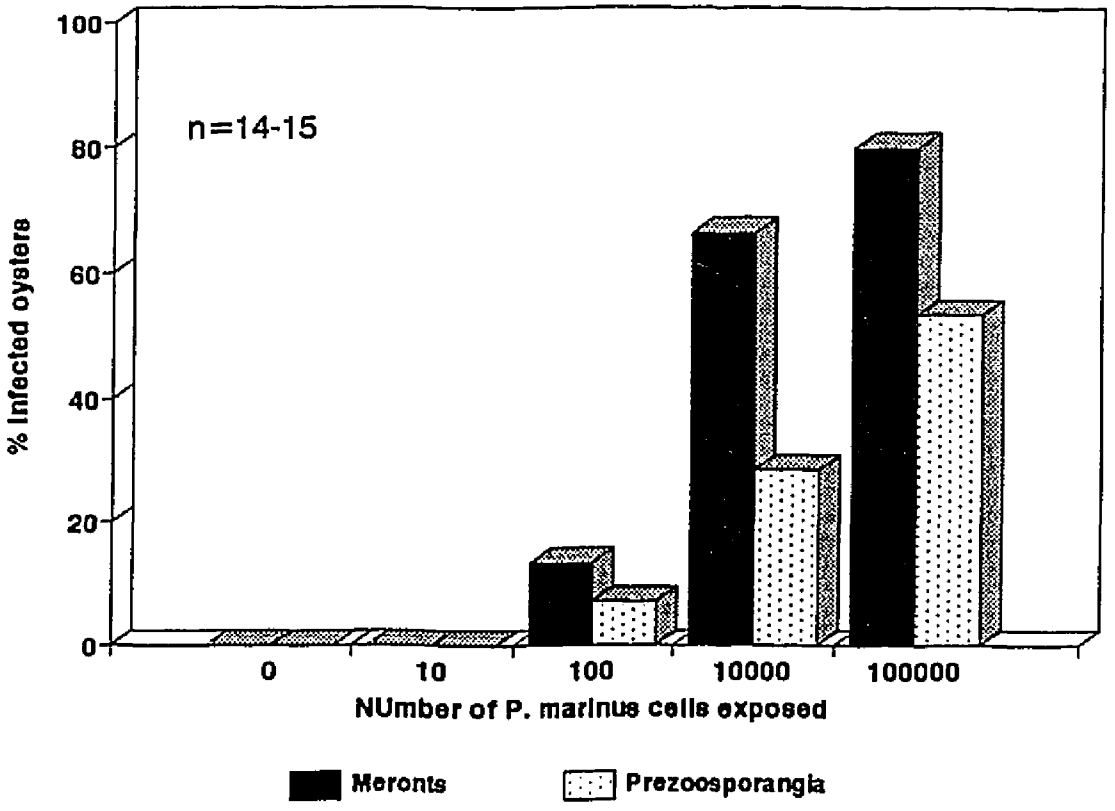
Mean THC in oysters at 10 and 15°C was significantly greater than in oysters at 25°C ($p < 0.05$) (Fig 9). However, oysters at higher temperatures had higher PG counts. Mean PG in oysters at 25°C was significantly greater than those at 10

and 15°C ($p < 0.0001$) (Fig 10). Both THC and PG were not affected by either salinity or the number of meronts injected into the oysters. There was no synergetic effect of temperature, salinity and infective particles concentration on THC or PG in oysters.

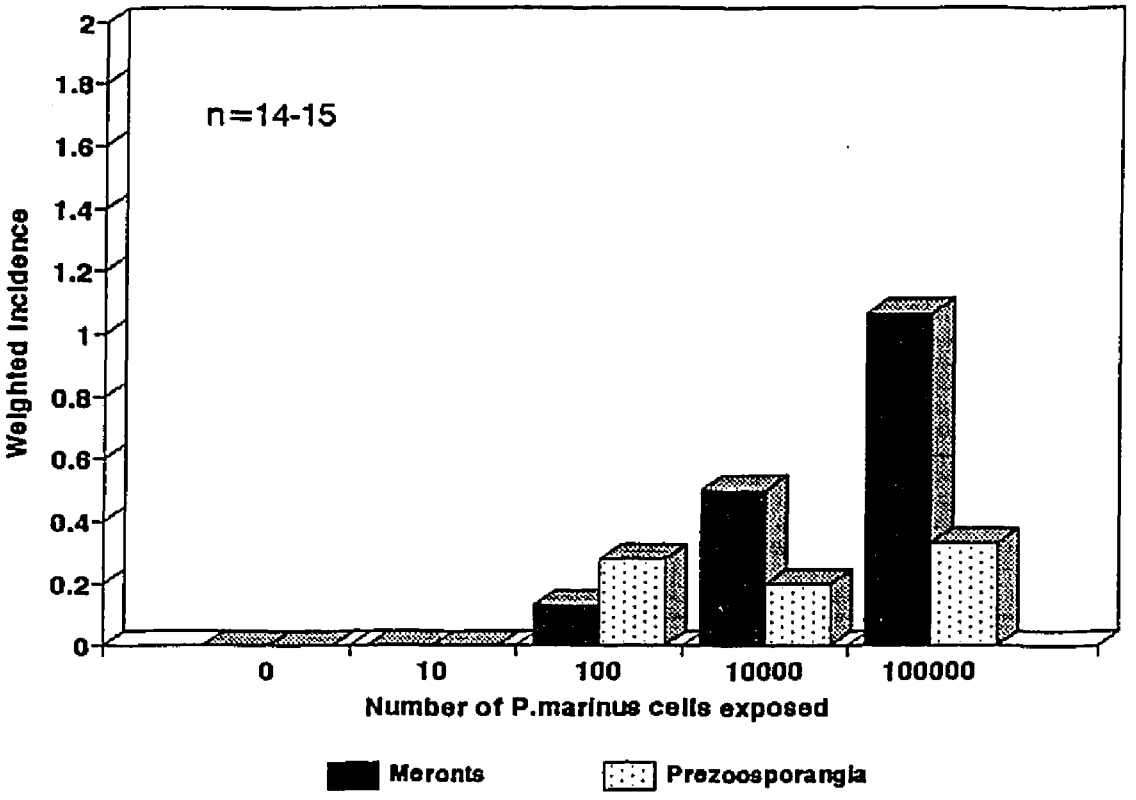
Mean serum protein concentrations were significantly higher in oysters for treatments at higher temperature (Fig 11a) ($p < 0.01$) and salinity (Fig 11b) ($p < 0.01$). Protein concentrations in oysters at 15 and 25°C were similar, but significantly higher than oysters at 10°C (Fig 11a). Also, protein concentrations in oysters at 20 ppt were significantly higher protein than oysters at 10 and 3 ppt (Fig 11b). It is surprising to note that infected oysters had significantly higher protein (Mean ± SE 1.85 ± 0.37mg/ml) ($p < 0.05$) compared to uninfected oysters (1.02 ± 0.16 mg/ml). The interaction of salinity and doses of *P. marinus* posted a significant effect on protein ($p < 0.01$). Lysozyme concentrations in oysters at 10°C was significantly greater ($p < 0.0001$) than in oysters at 15 and 25°C (Fig 12a). However, lysozyme concentrations decreased with decreasing salinity ($p < 0.0001$). Oysters at 20 ppt had the highest lysozyme concentration (Fig 12b). Both lysozyme and protein concentrations were not affected by the number of meronts injected into the oysters.

Figs 1a and 1b: Prevalence of *P. marinus* infection in oysters after 60 days post-challenge (Trial 1) (Fig 1a) and 90 days po


n=14-15
% Infected oysters
100
80
60
40
20
0
0
10
100
10000
100000
Number of P.marinus cells exposed
Meronts
Prezoosporangia


n=14-15
% Infected oysters
100
80
60
40
20
0
0
10
100
10000
100000
NUmber of P. marinus cells exposed
Meronts
Prezoosporangia

Figs 2a and 2b: Weighted prevalence of *P. marinus* infection in oysters after 60 days post-challenge (Fig 2a) and 90 days post-challenge (Fig 2b) with 0, 10, 10^2, 10^4, and 10^5 meronts and prezoosporangia.


n=14-15
Weighted Incidence
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
100
10000
100000
Number of P.marinus cells exposed
Meronts
Prezoosporangia

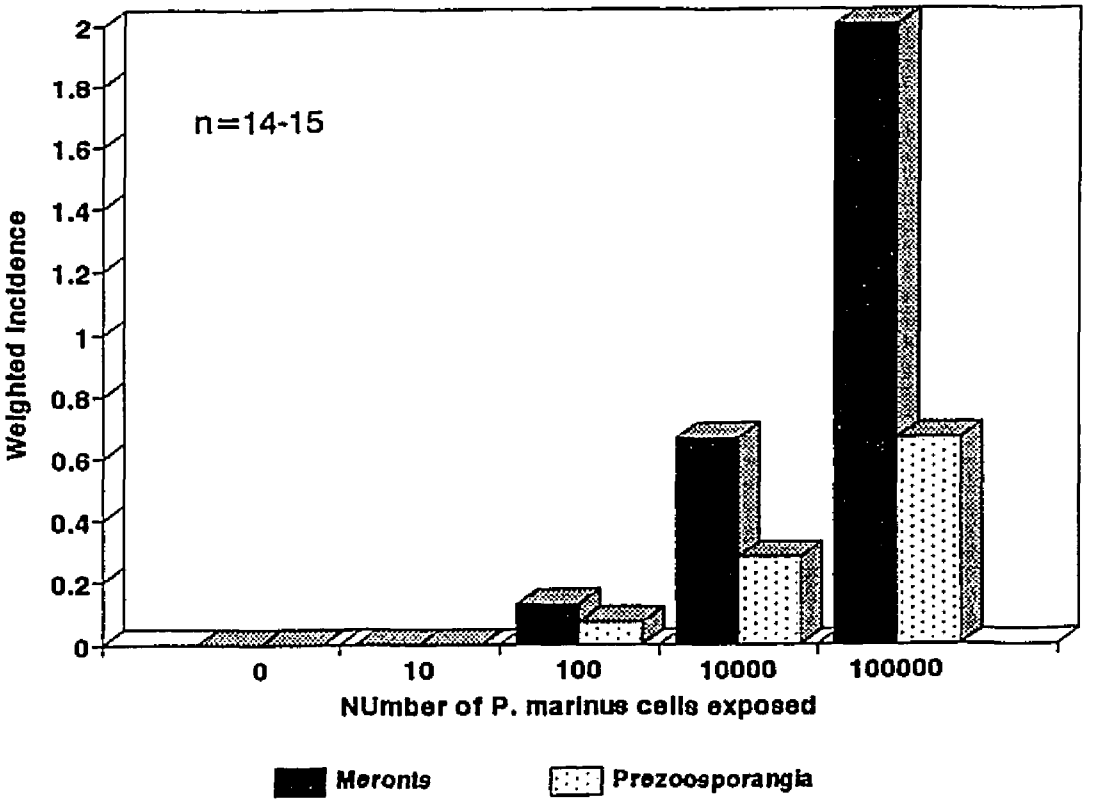

n=14-15
Weighted Incidence
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
100
10000
100000
NUmber of P. marinus cells exposed
Meronts
Prezoosporangia

Fig 3: Mean CI (± SE) in meront and prezoosporangia challenged oysters (Trial 1). Bars with different letters denote significance ($p < 0.05$).

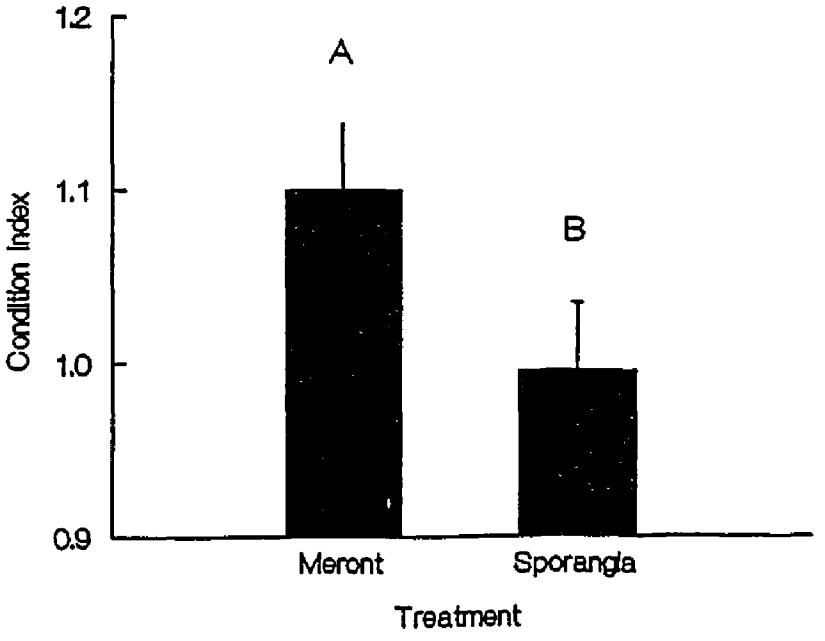
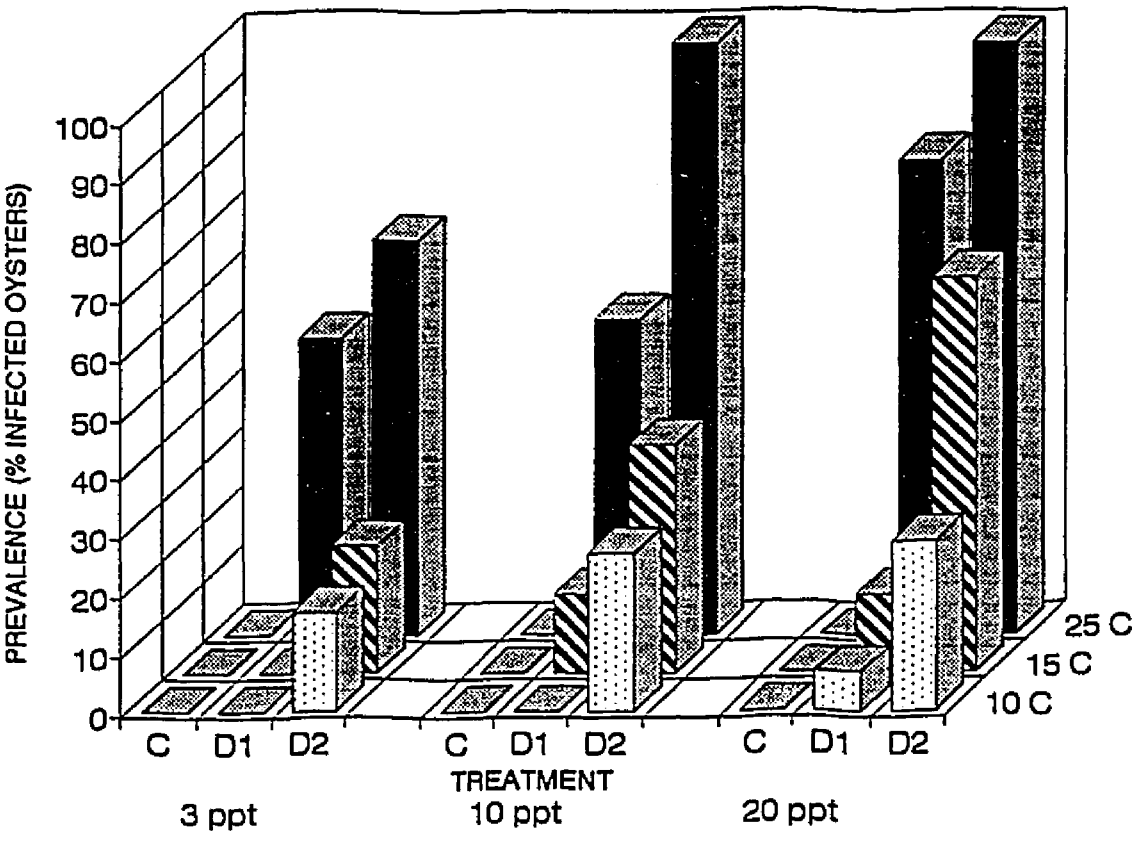
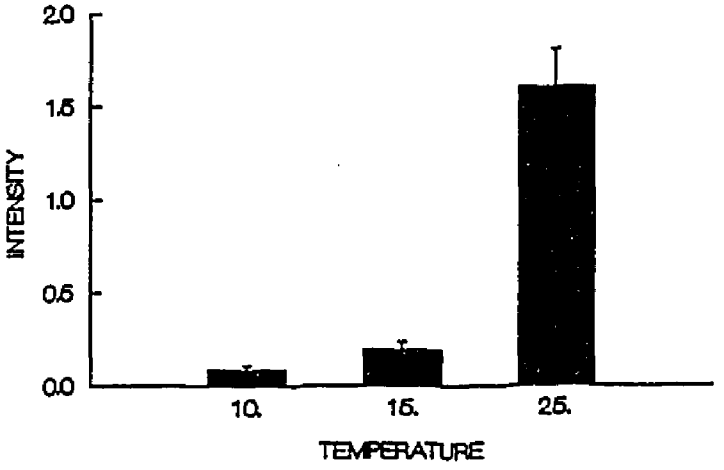

1.2
1.1
1.0
0.9
Condition Index
A
B
Meront
Sporangia
Treatment

Fig 4: Prevalence of *P. marinus* infection in oysters at 10, 15 and 25°C and 3, 10 and 20 ppt, challenged with 2.5 x 10^3 (D1), or 2.5 x 10^4 (D2) meronts.

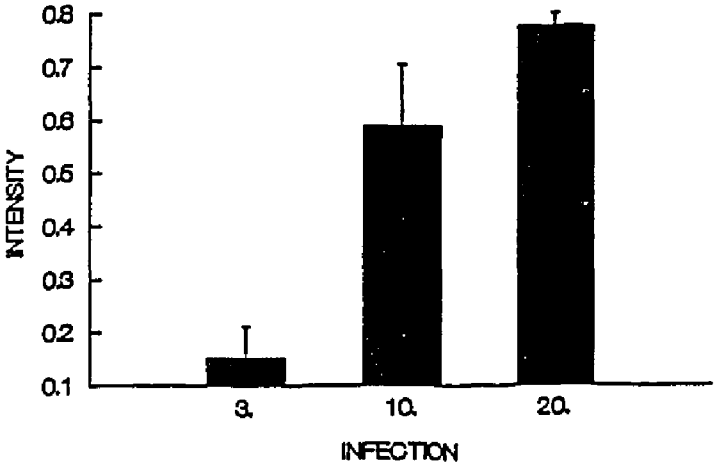

PREVALENCE (% INFECTED OYSTERS)
100
90
80
70
60
50
40
30
20
10
0
C D1 D2
C D1 D2
C D1 D2
TREATMENT
3 ppt
10 ppt
20 ppt
25 C
15 C
10 C

Figs 5a, 5b and 5c: *P. marinus* weighted prevalence in oysters at 10, 15 and 25°C (Fig 5a), at 3, 10 and 20 ppt (Fig 5b); and oysters challenged with 0, 2.5×10^3, or 2.5×10^4 meronts (Fig 5c). Bars with different letters denote significance ($p < 0.01$).

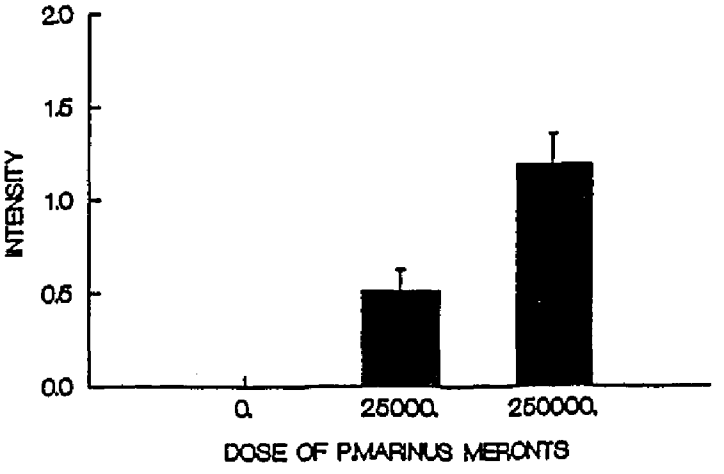
TEMPERATURE EFFECT



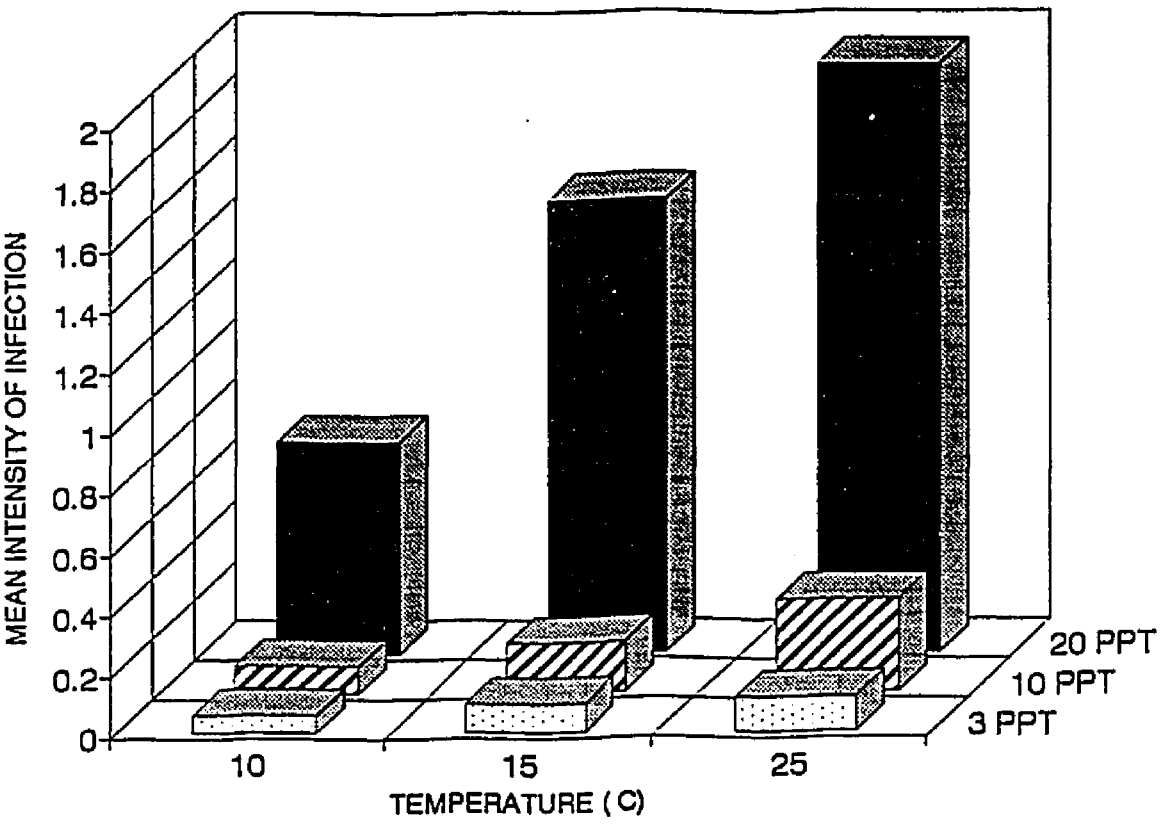
SALINITY EFFECT



EFFECT OF P.MARINUS



Figs 6a and 6b: Weighted prevalence of *P. marinus* infection in oysters at 10, 15 and 25°C and 3, 10 and 20 ppt (Fig 6a); at 10, 15 and 25°C challenged with control (0), Dose 1 (2.5×10^3) or Dose 2 (2.5×10^4) meronts.


MEAN INTENSITY OF INFECTION
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10
15
25
TEMPERATURE (C)
20 PPT
10 PPT
3 PPT

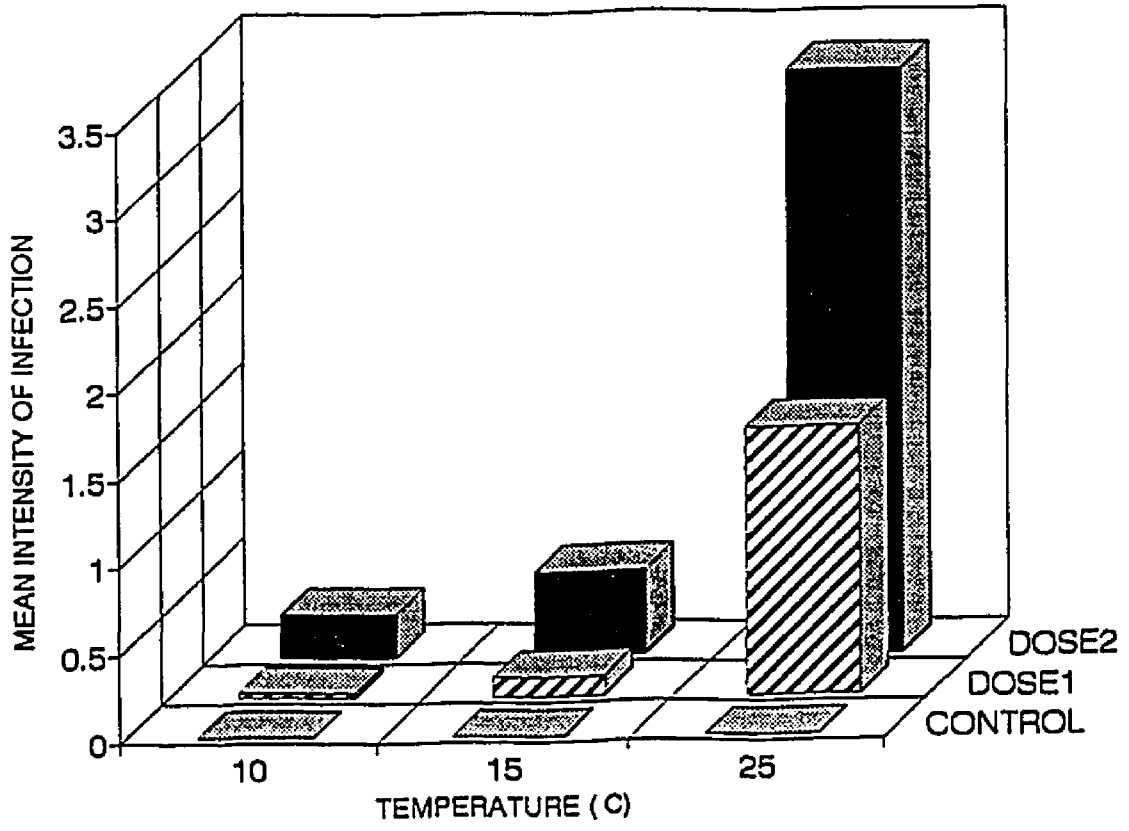
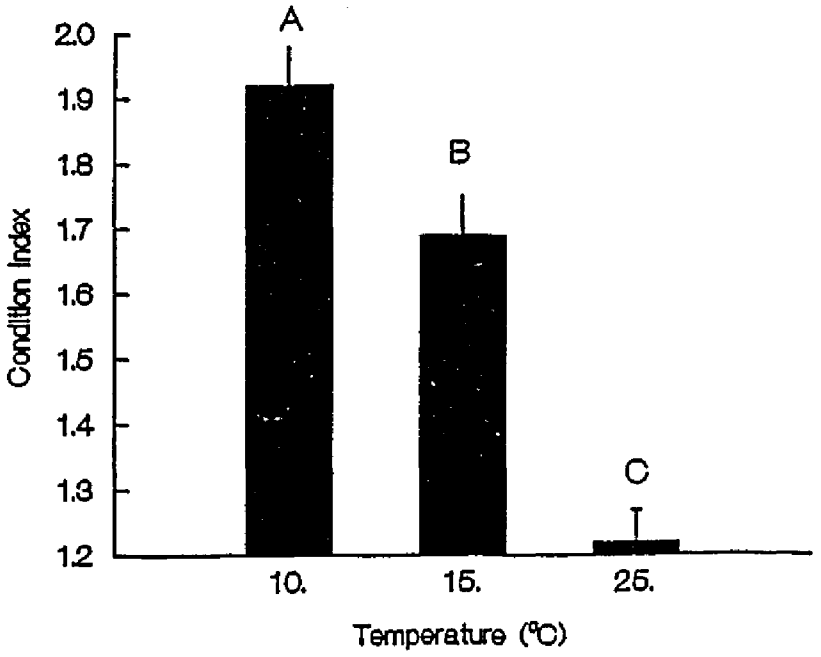

MEAN INTENSITY OF INFECTION
3.5
3
2.5
2
1.5
1
0.5
0
10
15
25
TEMPERATURE (C)
DOSE2
DOSE1
CONTROL

Fig 7: Mean CI (± SE) of oysters at 10, 15 and 25°C. Bars with different letters denote significance ($p < 0.0001$).

Fig 8: Mean CI (± SE) in infected and uninfected oysters. Bars with different letters denote significance ($p < 0.05$).


A
B
C
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
Condition Index
10.
15.
25.
Temperature (°C)

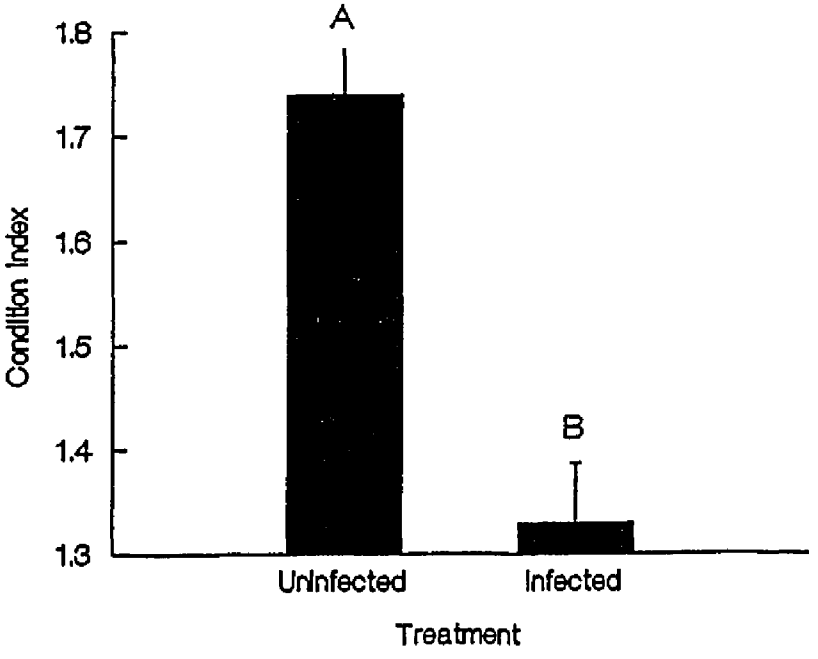
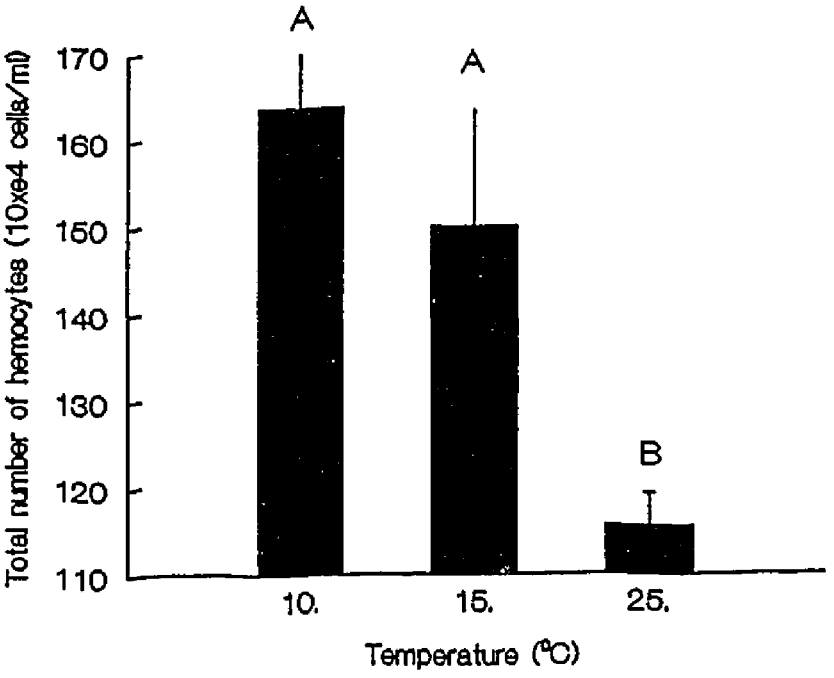
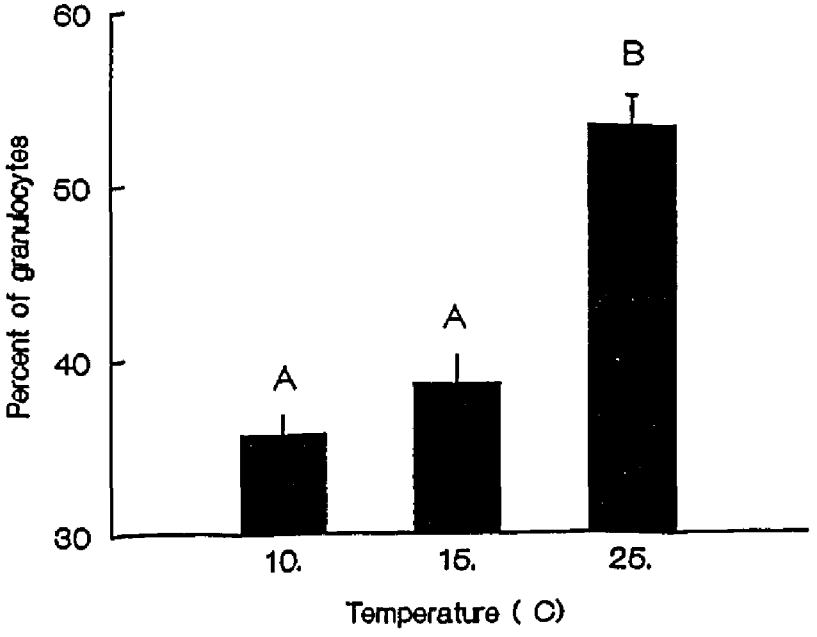

A
B
1.8
1.7
1.6
1.5
1.4
1.3
Condition Index
Uninfected
Infected
Treatment

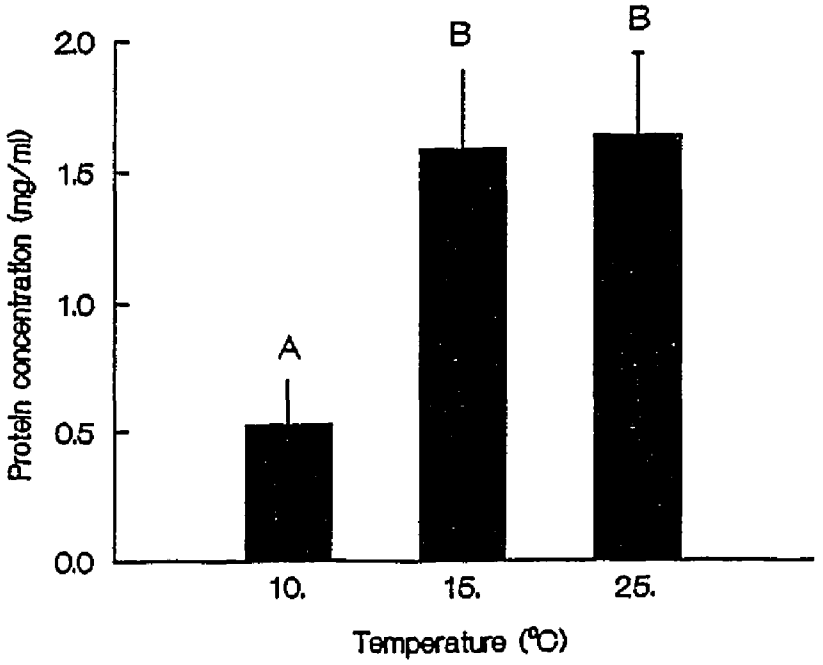
Fig 9: Mean THC (± SE) of oysters at 10, 15 and 25°C. Bars with different letters denote significance ($p < 0.05$).

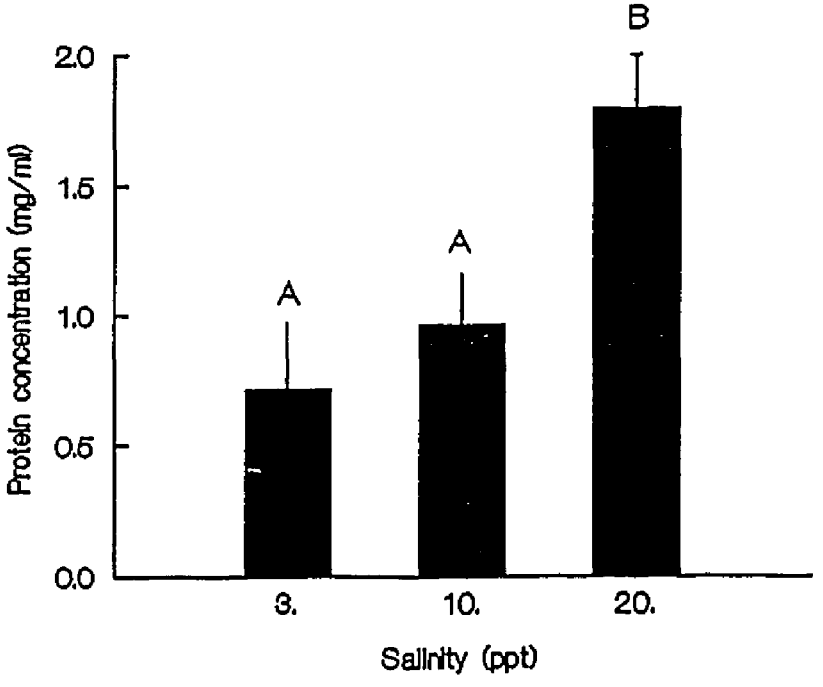
Fig 10: Mean PG (± SE) of oysters at 10, 15 and 25°C. Bars with different letters denote significance ($p < 0.0001$).


A
A
B
170
160
150
140
130
120
110
Total number of hemocytes (10xe4 cells/ml)
10.
15.
25.
Temperature (°C)

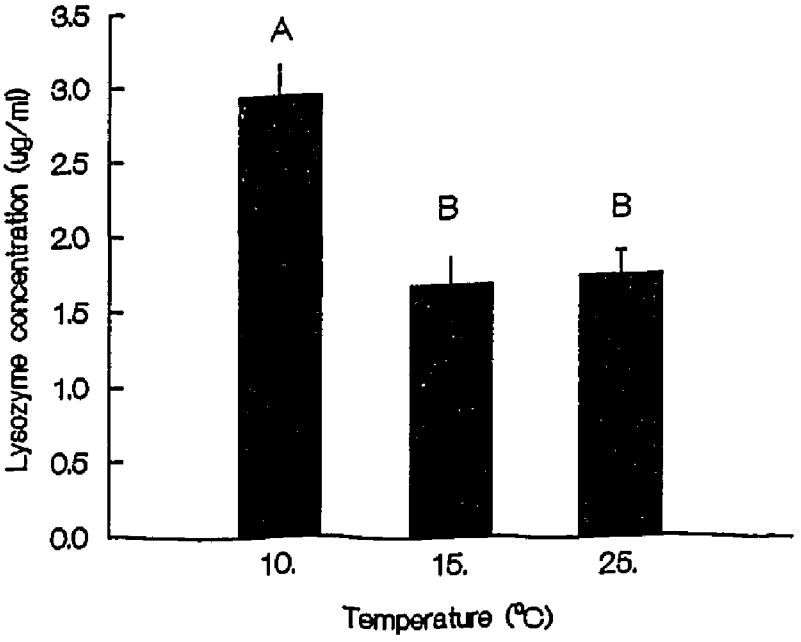

B
A
A
60
50
40
30
Percent of granulocytes
10.
15.
25.
Temperature (C)

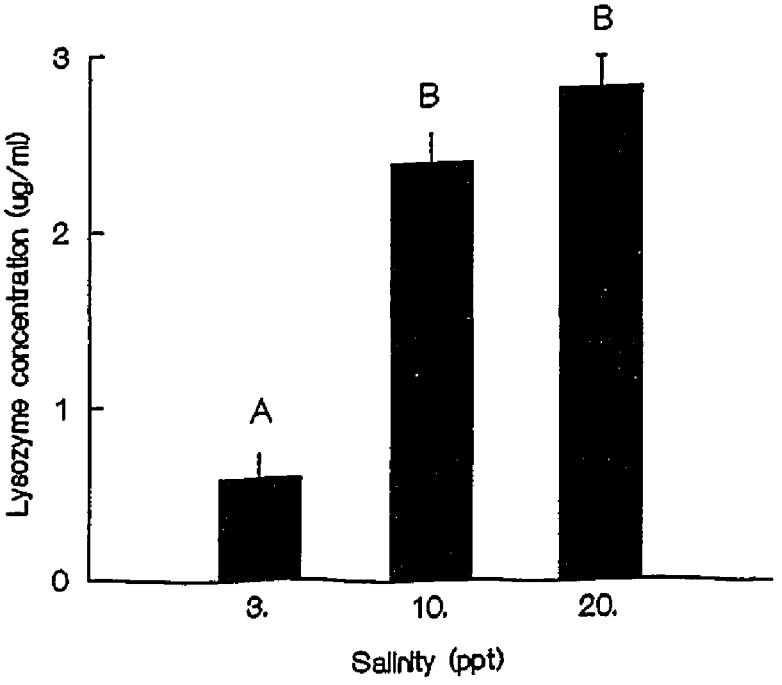
Figs 11a and 11b: Mean hemolymph protein concentration in oysters at 10, 15 and 25°C (Fig 11a) and 3, 10 and 20 ppt (Fig 11b). Bars with different letters denote significance ($p < 0.01$).


2.0
1.5
1.0
0.5
0.0
Protein concentration (mg/ml)
A
B
B
10.
15.
25.
Temperature (°C)


2.0
1.5
1.0
0.5
0.0
Protein concentration (mg/ml)
A
A
B
3.
10.
20.
Salinity (ppt)

Figs 12a and 12b: Mean hemolymph lysozyme concentration in oysters at 10, 15 and 25°C (Fig 12a) and 3, 10 and 20 ppt (Fig 12b). Bars with different letters denote significance ($p < 0.0001$).


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Lysozyme concentration (ug/ml)
A
B
B
10.
15.
25.
Temperature (°C)


3
2
1
0
Lysozyme concentration (ug/ml)
A
B
B
3.
10.
20.
Salinity (ppt)

DISCUSSION

Entry through filtration and/or feeding is presumed to be the main route or *P. marinus* transmission into oysters in nature. However, shell cavity inoculation has the advantage of challenging the oyster with a known quantity of meronts. The feeding method may be more comparable to natural conditions, but with the limitation to asses the exact number of meronts being exposed or entering the oyster tissue. *P. marinus* cells carried by water currents may attach on to gills and mantle when water passes through the shell cavity during filtration. *P. marinus* cells are quite sticky. Thus, shell cavity inoculation may be somewhat similar to natural conditions in which the inoculated infective cells have a direct contact with oyster tissue. Shell cavity and adductor muscle injection of meronts (> 10^4 meronts) produced greater infections, while feeding 10^7 meronts to oysters did not produce infections (Bushek 1994). Shell cavity injection seems to be more effective for artificial infection of oysters.

The findings of the dose response experiments reinforce previous findings that both meronts and prezoosporangia effectively transmit disease (Volety and Chu 1994) and clearly showed that meronts are more effective than prezoosporangia for *P. marinus* disease transmission in oysters under

laboratory conditions. A dose dependent response was demonstrated between number of *P. marinus* cells to which the oysters are exposed to and Dermo prevalence and intensity in the oysters (Figs 1a, 1b and 2a, 2b). Dose response in oysters to *in vitro* cultured *P. marinus* was examined by Bushek (1994) and obtained similar results. Mackin (1962) has suggested that mortality of oysters caused by Dermo infection is related to the dose of *P. marinus* cells. Mackin (1962) found that 10^2 *P. marinus* cells in minced tissue homogenate inoculated into oysters caused mortality in oysters within 37 days. Our results also indicate that the minimum number of *P. marinus* infective particles necessary to initiate infection in oysters is about 10^2 cells. However, in the present study, no mortality of oysters was noted during the experimental period. Only light infections were obtained in oysters exposed to doses of $<10^3$ *P. marinus* meronts (Figs 1a and 1b). Differences in infectivity seem to exist between "cultured" meronts and meronts isolated from infected oyster tissues ("wild"). More than 10^4 laboratory cultured meronts/oyster (Bushek 1994), and 10^6 meronts/oyster (La Peyre 1993) were required to initiate light infections in oysters, while fewer than 10^3-10^4 "wild" meronts produced moderate to heavy infections (Figs 6a and 6b). However, Gauthier and Vasta (1993) found that a two biweekly injections of 2.5×10^5 cultured meronts resulted in heavy infections in oysters after 4-5 weeks.

The results of the experiment examining synergetic effects of temperature, salinity and Dermo dose response in oysters indicate that temperature is the most important environmental factor influencing *P. marinus* infection in oysters. These results are consistent with previous field and laboratory findings (Soniat 1985, Fisher et al. 1992). It has been demonstrated that growth rates of *P. marinus in vitro* increased with increasing temperature (up to 28°C) (Chapter 4). This may contribute to the higher infection rate in oysters at higher temperatures.

P. marinus cell concentration is the next important factor (< temperature) in contributing to *P. marinus* infection in oysters. As suggested by Mackin (1962), in low salinity areas, increased fresh water input increases the flow of water out of the estuary and may dilute the infective particles. This would decrease the number of cells the oyster encounters, thus resulting in reduced *P. marinus* infection in these areas. Results from the present study support his view. Diluting the infective particles during peak infection season (summer-fall) by influx of fresh water or tidal flushing may alleviate Dermo infection in oyster population.

The concentration of *P. marinus* meronts in the water column during March - October 1992 can be as high as 19,000

meronts/lt in the upper Chesapeake Bay (Oxford, MD) (Dungan and Roberson 1993b). Filtration rate of oysters range from a few liters to ~34 l/hr (Galstoff 1964). Considering the concentration of meronts in water column, oysters are exposed to several million meronts daily during the spring and summer season. However, large numbers of *P. marinus* could be filtered and eliminated simultaneously through feces and pseudofeces or may be destroyed when passing through the digestive tract of the oyster. Active elimination of *P. marinus* cells by oysters through feces and pseudofeces has been observed by Bushek (1994). Although, more than 10^2 meronts and prezoosporangia may be necessary to induce infections in nature, once $10-10^2$ infective cells are trapped in the shell cavity, infection will occur. Despite high eliminating capability of particulate matter in oysters, chronic exposure to high levels of *P. marinus* cells may generate the heavy infections and mortalities in oysters observed in field studies (Bushek 1994).

High mortality in oysters recorded at 3 ppt when oysters were adjusted to a temperature of 25°C indicates that Maine oysters, apparently, could not tolerate salinities lower than 10 ppt and temperature higher than 15°C. Thus, habitat or genetic factors could influence the susceptibility of oysters to Dermo infection. Differences in susceptibility to *P. marinus* infection were observed between oyster populations

from Virginia, Texas, Maine and New Jersey (Bushek 1994). His studies have indicated that oysters from Virginia and Texas are less susceptible to *P. marinus* infections than Maine and New Jersey oyster populations, which are relatively naive. It was suggested that oyster populations from Texas and Virginia with more than 40 years of natural *P. marinus* exposure may have acquired partial resistance or decreased susceptibility compared to oysters from areas without *P. marinus*.

Although, increased salinity significantly enhanced *P. marinus* infection, salinity was less than temperature and salinity in influencing *P. marinus* prevalence in oysters. Fisher et al (1992) examined the disease progression of *P. marinus* infection in oysters collected from the Gulf of Mexico and maintained at different temperatures and salinities under laboratory conditions. Their results indicated that temperature was more influential than salinity in influencing *P. marinus* intensity and mortality of oysters. Previous studies indicated that infection prevalence and intensity were positively correlated with salinity (Mackin 1961, Chu et al. 1993, Soniat 1985, Soniat and Gauthier 1989, Craig et al. 1989, Gauthier et al. 1990, Paynter and Burreson 1991, Ragone and Burreson 1993). Low salinities may affect the physiology of the parasite. It has been suggested that at low salinities (< 12 ppt), the inability of cell volume regulation in cultured *P. marinus* cells contribute to their mortality

(Burreson et al. 1994). Ragone and Burreson (1993) reported increased survival of *P. marinus* infected oysters maintained at 6 ppt, compared to oysters at 20 ppt. Only light infections were observed in oysters below 10 ppt in the synergetic experiment in the present study. The high infection intensities at elevated salinities may be due to increased concentration and/or virulence of *P. marinus* cells at higher salinity, as suggested by Chu and Greene (1989) and Chu and La Peyre (1991). The physiological changes in oysters due to salinity changes has also been suggested to alter the susceptibility of the oysters to *P. marinus* infection (Scott et al. 1985). Thus, the increased infection seen in the present study at higher salinities may have been either due to increased susceptibility of the oysters and/or increased virulence of *P. marinus* at higher salinities. Although the effect of temperature, salinity and dose of *P. marinus* cells alone significantly influence the prevalence of Dermo infection in oysters, the interactive effect of these three factors (temperature, dose of *P. marinus* particles and salinity) on the prevalence of infection were found to be insignificant.

Soniat (1985) found a significant correlation between *P. marinus* infection intensity and interaction of temperature and salinity. The product of temperature and salinity, described as the interaction term, was more closely correlated with

weighted incidence than was temperature and salinity alone. In a separate study, Soniat and Gauthier (1989) found correlation between weighted incidence and salinity, but failed to detect a correlation between weighted incidence and the interaction of temperature and salinity. In both of their studies, no correlation was observed between temperature and weighted incidence. The lack of correlation between temperature and weighted incidence in Soniat and Gauthier's study (1989) may have been due to the low variability of the temperatures (10-30°C) in the Louisiana area during the period of study. Results of the present study also indicate a significant interaction between temperature and salinity ($p < 0.05$, Fig 6a). The interaction of temperature and dose of *P. marinus* meronts was also significant. However, the interaction of salinity and dose on *P. marinus* intensity in oysters was not significant. The interaction of temperatures and salinities, as well as between temperatures and *P. marinus* dosage intensified infections.

Similar to our previous findings (Volety and Chu 1994), in the dose experiment, CI of oysters (Trial 1) infected by prezoosporangia was significantly lower, compared to that of oysters infected by meronts. CI is insignificantly different between infected and uninfected oysters, as most of the oysters in the dose experiment were lightly infected. However, CI of infected oysters was significantly lower than

uninfected oysters in the synergetic effect experiment. This may be because most of the oysters at higher temperatures, salinities, and doses of *P. marinus* cells were moderately to heavily infected (particularly at > 15°C, 20 ppt and 10^4 meronts) (intensity 2 - 3.5; Fig 6a and 6b). Reduced CI in heavily infected oysters was reported by Paynter and Burreson (1991) and Dittman (1993). The increased (challenge) number 2.5×10^4 meronts of *P. marinus* cells may also exert increased energy demands on the oysters.

Temperature significantly affected CI of oysters. Oysters maintained at lower temperatures (10 and 15°C) had significantly higher CI compared to oysters at 25°C. The lower CI values at 15 and 25°C may be due to higher metabolism at elevated temperatures. The higher CI at lower temperatures may be due to lower prevalence and intensities of infection and support previous findings reported by Chu and La Peyre (1993a). In addition, high temperatures may also be stressing the oysters adapted to a cold climate (Damarsicotta River, Maine). Salinity, however, had no significant influence on CI of oysters. Similar results were reported by Crosby and Roberts (1990) and Paynter and Burreson (1991).

Higher THC and PG were observed in oysters at higher temperatures (Chu and La Peyre 1993a). Similar to previous findings, temperature significantly affected THC and PG in

oysters in the present study. Although, higher PG were observed at higher temperatures in the present study, THC was unexpectedly higher at lower (10 and 15°C) than at higher temperatures (25°C). This is in contrast to previous findings (Chu and La Peyre 1993a). The oysters used in this study were obtained from an area of low temperature, hence higher temperatures such as the one in this experiment (25°C) may have stressed the oysters, resulting in lower THC in oysters despite the gradual acclimation of oysters to experimental conditions. Both salinity or dose of *P. marinus* cells did not affect THC and PG in oysters. The significant interaction effect of temperature and *P. marinus* infection on PG may suggest a pathological effect.

It is surprising to note that the protein concentration exhibited a trend opposite to that of CI. Contradictory to the findings from the present study, higher protein concentrations in oysters at low temperatures (10°C) compared to oysters at higher temperatures (15-25°C) were observed by Chu and La Peyre (1993a). High metabolic rate at high temperatures was cited as the reason for the reduction of plasma protein in their study. The reasons for the high protein concentrations at high temperatures (Fig 12a) in the present study is difficult to explain. Higher growth rates were observed in oysters at higher salinities (Paynter and Burreson 1991). Thus, high protein concentrations at high

salinities observed in the present study are not surprising. Earlier studies by Chu et al. (1993) and Fisher and Newell (1986) indicated that salinity (6-36 ppt) did not effect hemolymph protein within the 4-5 weeks experimental period. The experimental period, however, in this experiment was more than 16 weeks (including acclimation time), hence, the results could have been different. Since high temperatures and salinities affected protein concentration in oysters, the higher protein concentration in infected oysters compared to uninfected oysters may be due to the effect of temperature and salinity and not due to parasitism. Also, most of the oysters in this experiment were only lightly infected (WI=0.2-1.2).

Negative correlation was observed between lysozyme and temperature (Chu and La Peyre 1993a) and lysozyme and salinity (Chu et al. 1993). In the present study, lysozyme concentration in oysters was higher at 10°C compared to oysters at 15 and 25°C, thus supporting previous findings. The low lysozyme concentration in oysters at 3 ppt suggests that this salinity may be too stressful for the Maine oysters, which were from a habitat of salinity 32-33 ppt. No significant differences in lysozyme concentrations were found between infected and uninfected oysters. Similar results were reported by Chu et al. (1993).

In summary, environmental factors such as temperature and

salinity modulate host-parasite interactions. Effect of temperature in combination with dilution of infective particle with fresh water influx and/or decreased salinity may result in low Dermo infections in oysters in the field. Low prevalence and intensity of *P. marinus* infection in oysters at low temperature and salinity regimes may be explained by the increased mortality of *P. marinus* cells at low temperatures and salinity. Although no synergetic effects existed between temperature, salinity and dose of *P. marinus* particles on Dermo infection in oysters, all three factors undoubtedly effect the physiology of the host, the parasite as well, and subsequent Dermo infection in oysters. For example, temperature and salinity affected the THC, PG, protein and lysozyme concentrations. Information gained from understanding the effect of environmental factors on disease processes could potentially be used in effective management strategies in preventing epizootics of oyster populations.

CHAPTER 4

SUPPRESSION OF CHEMILUMINSCENCE OF EASTERN OYSTER (*Crassostrea virginica*) HEMOCYTES, BY THE PROTOZOAN PARASITE *Perkinsus marinus*.

ABSTRACT

Experiments were conducted to determine the ability of the protistan parasite, *Perkinsus marinus*, to suppress/inhibit chemiluminescence of hemocytes from the eastern oyster, *Crassostrea virginica*. Luminol enhanced chemiluminescence (CL, counts per minute) was used to measure the production of reactive oxygen intermediates (ROI) generated by oyster hemocytes using zymosan as a stimulant. To determine whether *P. marinus* suppresses ROI evoked from zymosan-stimulated hemocytes, live or heat killed *P. marinus* in filtered estuarine water (YRW) (salinity = 20ppt) were added to (1) zymosan-stimulated hemocytes after CL reached its peak, or (2) hemocytes at the same time as zymosan, and reduction of CL responses were recorded. In both tests, controls received estuarine water only. Live *P. marinus* meronts significantly suppressed ROI production by zymosan-stimulated hemocytes. The suppression of ROI production was dose dependent ($p < 0.05$). Suppression of ROI production from zymosan-stimulated hemocytes by heat killed *P. marinus* was significantly less than live *P. marinus* ($p < 0.001$). Similarly, CL of hemocytes was reduced, though not significantly ($p > 0.05$) when hemocytes were exposed to YRW preincubated with *P. marinus*. When *P. marinus* meronts were used as a stimulant, no CL response was elicited. Results of this study suggest that *P. marinus* cells are able to suppress ROI release from oyster

hemocytes, thus evading this component of the host's defense.

INTRODUCTION

Heavy mortalities of oysters in Chesapeake Bay and on the east coast of the United States caused by the protozoan parasite, *Perkinsus marinus*, have been well documented (Andrews 1988, Burreson 1989) in recent years. The physiopathologic effects of the disease organism on the oysters have been studied extensively (Paynter and Burreson 1991, Chu and La Peyre 1993a, Chu et al. 1993), however, very little is known about the evasive mechanisms of the parasite in escaping the host's defense mechanisms.

Defense mechanisms in vertebrate phagocytes involve complex processes including the production of toxic free oxygen radicals by the respiratory burst (Allen et al. 1972), and other enzymatic processes (Thomas et al. 1988). When vertebrate macrophage membranes are stimulated by foreign particles or organisms, stimulation of NADPH oxidase (Takeshige and Minakami 1987, Jones et al. 1982), and activation of the hexose monophosphate pathway occurs. This process is accompanied by production of toxic reactive oxygen intermediates (ROI) such as OH^-, H_2O_2, $^1O_2$ and O_2^- which may be involved in cellular killing. Also, H_2O_2 along with myeloperoxidase and halide ions results in the formation of hypohalites and singlet oxygen which are microbicidal (Chung and Secombes 1988, Schlenk et al. 1991). Similar mechanisms

were noted in invertebrates such as the shore crab, *Carcinus maenas* (Bell and Smith 1993), sea urchin, *Strongylocentrotus nudus* (Ito et al. 1992) and molluscs, *Patinopecten yessoensis* (Nakamure at al. 1985), *Lymnea stagnalis* (Dikkeboom et al. 1987), *Planorborius corneus, Helix aspera* (Dikkebbom et al. 1988), *Biomphalaria glabrata* (Shozawa 1986), *pecten maximus* (Le Gall et al. 1991), *Crassostrea gigas* and *Ostrea edulis* (Bachere et al. 1991), and *Crassostrea virginica* (Larson et al. 1989, Fisher et al. 1990, Anderson et al. 1992b). The microbicidal activity associated with the production of ROI has been discussed by many authors (Allen et al. 1972, Adema et al. 1991, Horan et al. 1982, Welch 1980, Baboir et al. 1973). Release of ROI production coincided with phagocytosis of zymosan particles and chemiluminescence activity in *Lymnaea stagnalis* (Adema et al. 1991), suggesting an association between phagocytosis, ROI production and chemiluminescence. Production of both ROI and hydrogen peroxide inside the phagosomes of *L. stagnalis* hemocytes has also been documented (Dikkeboom et al. 1987). Luminol-enhanced CL has been used to measure ROIs production related to phagocytic activity in oyster species, *C. gigas* and *O. edulis* (Bachere et al. 1991) and *C. virginica* (Fisher et al. 1990, Anderson et al. 1992b). Involvement of the myeloperoxidase system in the production of ROI has been demonstrated in the common mussel, *Mytilus edulis* (Schelnk et al. 1991) and in *C. virginica* (Austin and Paynter, University of Maryland, personal communication).

Although invertebrates lack a complex immune system like that of vertebrates, they posses an effective defense system comprising cellular (Fisher 1988, Feng 1988) and humoral activities (Chu 1988). Hemocytes comprise a primary line of defense in molluscs and are responsible for activities such as inflammation, wound repair (Fisher 1988), phagocytosis and encapsulation (Fisher 1988, Sminia et al. 1987). Phagocytosis and degradation of *P. marinus* meronts have been demonstrated using transmission electron microscopy (La Peyre 1993, Bushek 1994). Stimulation of oyster hemocytes with *P. marinus* meronts, however, did not elicit any CL response (La Peyre 1993, Chu and Volety, unpublished results). This suggests that either *P. marinus* meronts are degraded by processes not mediated by ROI and/or *P. marinus* may be able to suppress, inhibit or scavenge the ROI released by the host hemocytes. Therefore, this study was conducted to investigate the possible suppression or inhibition of host's ROI production by *P. marinus*.

MATERIALS AND METHODS

Oysters and hemolymph collection:

Hemolymph was withdrawn from oysters (2.5 - 3") collected from the Rappahannock River, Virginia. One ml of hemolymph was collected from the adductor muscle of individual oysters using

a 27 gauge needle. Hemolymph was pooled and hemocyte concentration in the hemolymph was adjusted to 1 x 10^6 cells/ml for all CL assays in this study.

P. marinus:

P. marinus meronts were cultured according to Gauthier and Vasta (1993). The parasite has been subcultured for over 30 generations in our laboratory. The parasites were washed twice and resuspended in 1 µm filtered estuarine water (York River Water, YRW) at a concentration of 60 x 10^6 cells/ml. For the dose response study, *P. marinus* cells were adjusted to the appropriate concentrations using YRW. Both live and heat killed (100°C for 15 minutes) *P. marinus* were washed and resuspended in YRW at the aforementioned concentration.

Zymosan:

Zymosan (Sigma, USA) particles were used as a stimulant for the oyster hemocytes. Zymosan particles were suspended in YRW at a concentration of 10 mg/ml, heated for 30 min at 100°C, washed twice and resuspended in YRW at a concentration of 1 mg/ml.

Chemiluminescence assay:

Chemiluminescence (CL) was measured in a Beckman LS-3133T liquid scintillation counter in an out-of-coincidence mode at room temperature (22-23°C). Luminol was prepared according to

Scott and Klesius (1981). Five hundred µl hemolymph samples (N=3 to 4) and 500 µl luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, 1:500 dilution) in YRW in triplicates, unless otherwise stated, were aliquoted into plastic scintillation vials and baseline CL levels (counts per minute, CPM) were noted for 2-3 cycles (15-30 minutes). Five hundred µl of zymosan suspension were then added to the hemocyte mixture and CL responses recorded. Controls received 500 µl of YRW instead of zymosan. CL counts were plotted against time, and the area under the CL curve after the addition of zymosan/*P.marinus* was calculated using a Numonics 2400 digitiser and expressed as counts per minute (CPM).

Experiments:

1. Dose related response of hemocyte CL to *P. marinus*:

P. marinus cells were adjusted to a concentration of 7.5, 15, 30 and 60 x 10^6 cells/ml in YRW (1:7.5, 1:15, 1:30 and 1:60 hemocyte: *P. marinus* ratio). Five hundred µl of hemolymph and 500 µl luminol solution were placed in plastic scintillation vials and background CL was measured. Production of ROI was initiated by the addition of 500 µl of zymosan (500 µg) and 500 µl of *P. marinus* (3.75, 7.5, 15 or 30 x 10^6 cells) suspension. For each sample, counts were measured for 2 to 3 hours (8 - 12 cycles), 15 minutes/cycle. Triplicate samples were analyzed for each *P. marinus* dose used.

2. Suppression of CL by *P. marinus*:

Five hundred µl of hemocyte and luminol mixture (N=4) were aliquoted into scintillation vials, and the CL base line activity was measured for 2-3 cycles (8 minutes/cycle). Five hundred µl of zymosan was then added to the hemocyte mixture. When CL response reached its peak, 0.5 ml of a live or heat killed *P. marinus* meront suspension (30 x 10^6 cells) was added to the zymosan-stimulated hemocytes and reduction of CL activity was measured. Counts were conducted on each sample for 2 to 2.5 hours (15 - 18 cycles), 8 minutes/cycle. Zymosan-stimulated hemocytes added with 500 µl of YRW served as non-*P. marinus* controls. The second control (blank) received 500µl of YRW instead of zymosan.

3. Inhibition of CL by *P. marinus*:

Similar to trial 1 (N=4), the same concentrations of hemocytes, parasites and zymosan were used, with the exception that zymosan and *P. marinus* were added to the hemocyte suspension at the same time.

4. Effect of *P. marinus* secretions on hemocyte CL:

Cultured meronts were washed twice with YRW under sterile conditions, resuspended in YRW at a cell density of 60 X 10^6 cells/ml (N=6) and incubated at 25°C for 48 hrs. The cell suspensions were then centrifuged at 12000 x g for 10 minutes and the supernatants saved. Hemocytes in 500 µl plasma were

exposed to 500µl of *P. marinus* incubated YRW (supernatant, PMYRW). Controls were exposed to YRW. Zymosan was added to the hemocyte suspensions immediately following the addition of YRW and PMYRW and CL responses recorded. The second control (blank) received YRW instead of zymosan. Acid phosphatase activity of the PMYRW and YRW was assayed using a colorimetric assay (Sigma Diagnostics) based on the release of p-niitrophenol and inorganic phosphate from p-nitrophenyl phosphate by the enzyme (Andersch and Szczypinski 1947).

STATISTICAL ANALYSES

To compare the areas under the CL curve between treatments of (i) Zymosan stimulated hemocytes exposed to different doses of *P. marinus* and (ii) heat-killed, live *P. marinus* exposed, and control hemocytes in both suppression and inhibition assays, One-Way analysis of variance was used. Paired t-test was used to determine the differences between PMYRW and YRW incubated hemocyte CL and acid phosphatase activities in PMYRW and YRW. Differences were considered statistically significant if $p < 0.05$. All the experiments were repeated 3 - 4 times to determine the reproducibility.

RESULTS

Since results of all the experiments showed a similar trend,

the typical results of individual experiments are reported here.

P. marinus suppressed the CL activity of oyster hemocytes in a dose dependent manner (Fig 1). No significant reduction in CL was observed compared to controls when hemocytes were exposed to 3.75×10^6 *P. marinus* cells. However, suppression of the CL response was significant when hemocytes were exposed to 7.5, 15 and 30×10^6 *P. marinus* cells. (Fig 1).

The reduction of CL was significantly higher ($p < 0.05$) in hemocytes exposed to live *P. marinus* as compared to heat killed *P. marinus* or controls (Fig 2) when *P. marinus* cells were added to the hemocytes at their peak CL response. However, the difference in CL response between control hemocytes and hemocytes exposed to heat-killed *P. marinus* was insignificant ($p > 0.05$).

Similar results were demonstrated when zymosan and live or heat killed *P. marinus* were simultaneously added to the hemocytes (Fig 3). A significant decrease in CL activity in live *P. marinus* exposed hemocytes ($p < 0.05$) was noted, as compared to heat-killed and non-*P. marinus* exposed controls. Again, no significant differences in CL were observed between heat-killed *P. marinus* exposed and control hemocytes. No CL response was elicited by hemocytes when *P. marinus* meronts

were used as stimulant.

The CL response (mean ± 1 SE) of hemocytes exposed to non-PMIYRW (control) was higher (49685 ± 1717 cpm) than hemocytes exposed to PMIYRW (45178 ± 2266 cpm), however, the differences were statistically insignificant ($p < 0.15$). The acid phosphatase concentration (mean ± 1 SE) in PMIYRW (0.101 units/ml) was significantly higher ($p < 0.001$) than the controls (0 ± 0).

Fig 1: Dose response of oyster hemocyte CL to *P. marinus* meronts. Treatments 1, 2, 3, 4 and 5 = YRW, 3.75, 7.5, 15, and 30 x 10^6 *P. marinus* cells/5 x 10^5 hemocytes. Mean CPM of triplicate samples ± 1 SE. The same letters above the bars denote lack of significance ($p > 0.05$).

Dose response of P.marinus on CL of hemocytes

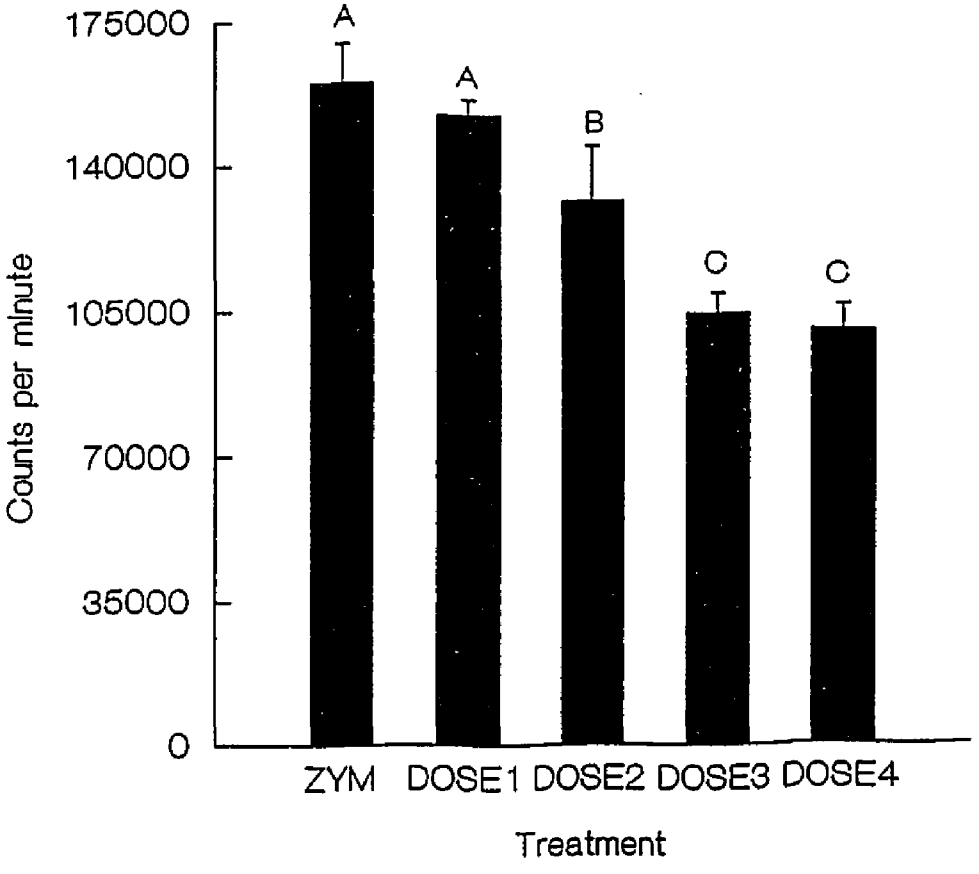


Fig 2: Suppression of zymosan-induced CL in oyster hemocytes by *P. marinus*. (HKP = heat-killed *P. marinus*; LP = live-*P. marinus*; ZYM = zymosan). *P. marinus* cells were added to hemocytes at their peak CL response. Mean CPM of four samples ± 1 SE. The same letters above the bars denote lack of significance ($p > 0.05$).

Inhibition of hemocyte CL by P. marinus

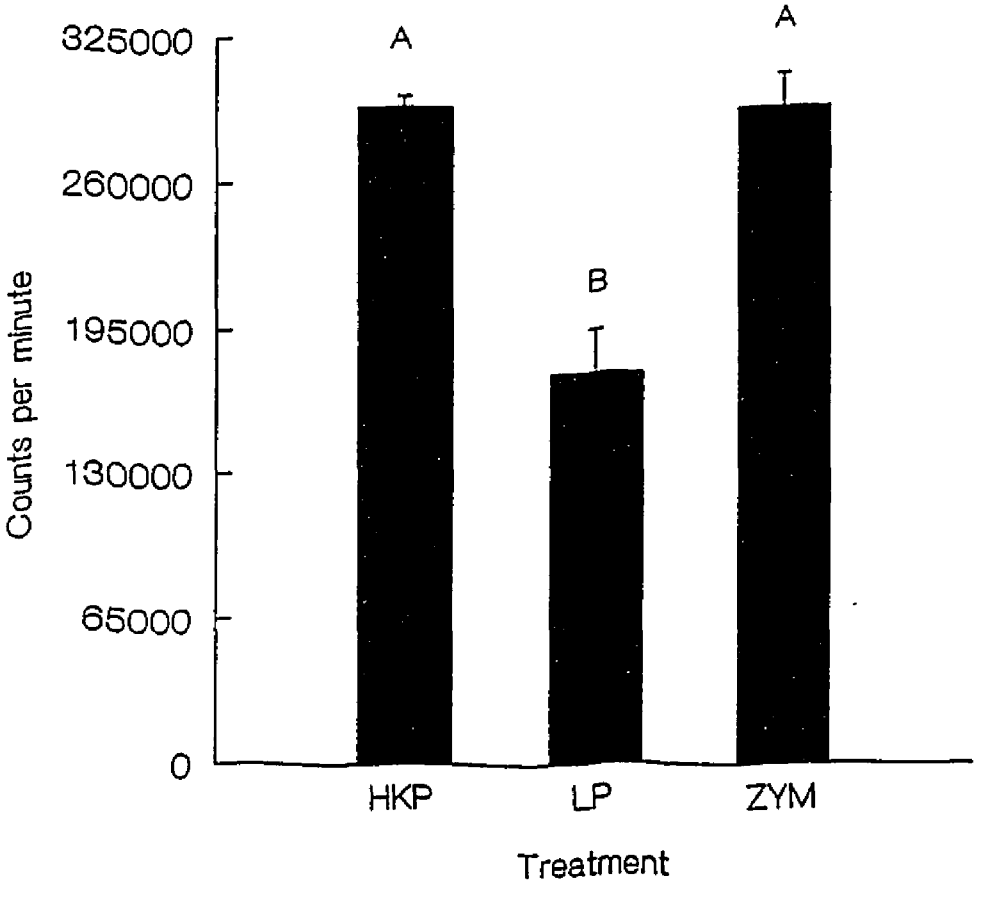
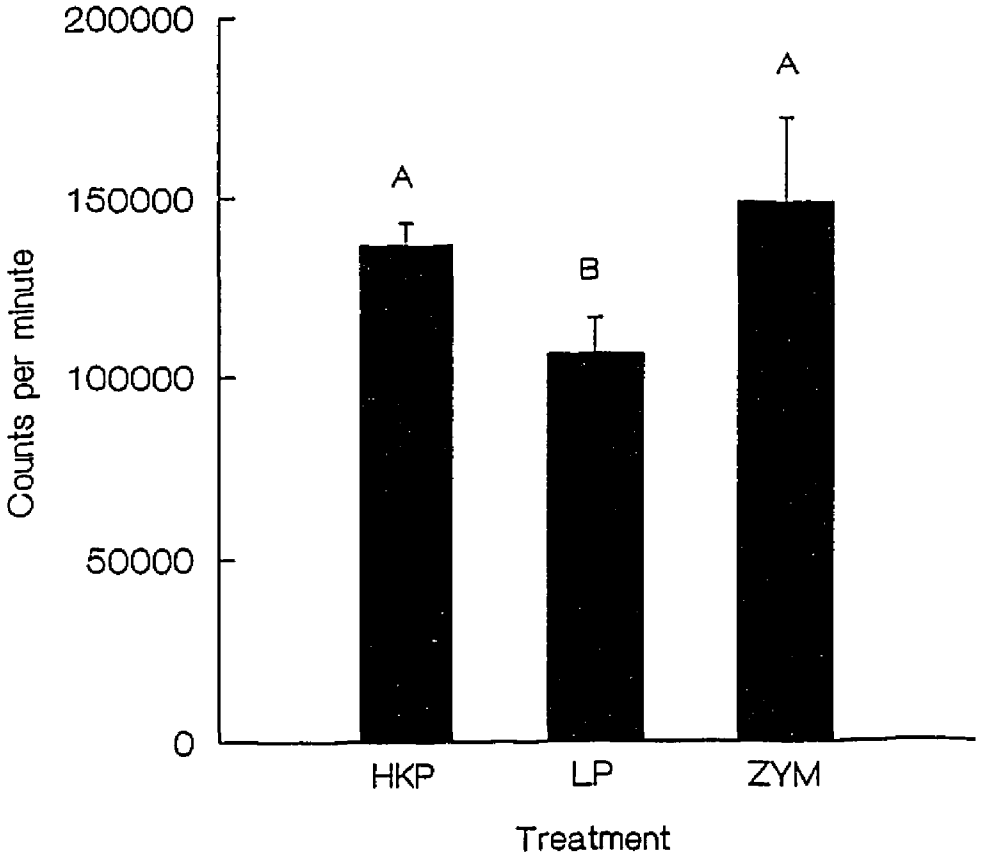


Fig 3: Inhibition of zymosan-induced CL in oyster hemocytes by *P. marinus*. (HKP = heat-killed *P. marinus*; LP = live-*P. marinus*; ZYM = zymosan). Mean CPM of four samples ± 1 SE. The same letters above the bars denote lack of significance ($p > 0.05$).

Suppression of oyster hemocyte CL by P. marinus



DISCUSSION

Results of all four experiments indicated that exposure of zymosan-stimulated hemocytes to live *P. marinus* or to the extracellular products of *P. marinus* results in suppression and/or inhibition of ROI production by oyster hemocytes. Certain protozoan parasites employ mechanisms such as active entry into host cells, entry into the host cells without triggering the respiratory burst, and suppression and/or inhibition of ROI production by phagocytes (Mauel 1984, Moulder 1985, Bogdan et al. 1990, Hall and Joiner 1991). *Leishmania spp.* enter the host macrophages by triggering a receptor and causing internalization, but not stimulating the respiratory burst (Russel and Talamas-Rohana 1989). Lypophosphoglycan in cell membranes of *Leishmania* promastigotes inhibits the respiratory burst of monocytes, possibly by having an inhibitory effect on protein kinase C (McNeely and Turco 1987).

Electron microscopic studies by La Peyre (1993) and Bushek et al. (1994) indicated that the oyster hemocytes are able to recognize and phagocytose freshly isolated and laboratory cultured *P. marinus* cells, but caused only limited degradation of the parasite in the hemocytes. It was also noted that no CL response was observed from hemocytes of either American (*C. virginica*) or Pacific (*C. gigas*) oysters

when exposed to *P. marinus* merozoites (La Peyre 1993). These results suggest that intracellular killing of *P. marinus* may not be mediated by toxic metabolites. The degradation of *P. marinus* by oyster hemocytes may be mediated by enzymatic factors such as the lysosomal enzymes. Presence of lysosomal enzymes in molluscs has been documented (Sminia and Barendsen 1980, Cheng and Rodrick 1975, Yoshino 1988). However, the exact mechanism involved in intracellular killing of *P. marinus* by oyster hemocytes remains unclear. The lack of any inhibition of ROI production by zymosan-stimulated hemocytes when exposed to heat killed *P. marinus* may suggest that denaturation of the ROI suppressor(s) occured in the parasite upon heating (Figs 2 and 3). Similar results were reported by Le Gall et al. (1991) when zymosan-stimulated *P. maximus* hemocytes were exposed to live and heat killed rikketsiales-like organisms (RLO). Their study demonstrated that exposure of *P. maximus* hemocytes to live RLO produced a greater suppression (45-74%) in CL activity compared to heat killed RLO (51-58%).

Investigations of Yoshino et al. (1993) revealed that the extracellular cysteine proteinase(s) of *Schistosoma mansoni* degrade high molecular weight hemolymph proteins of the host, *B. glabrata*. The YRW preincubated with *P. marinus* may contain extracelluar products (eg. acid phosphatase, catalase, superoxide dismutase, glutathione peroxidase, aminopeptidase)

from the parasite responsible for suppression/inhibition of ROI production by oyster hemocytes. Anti-oxidant enzymes, such as superoxide dismutase, catalase (Weiss et al. 1987) and glutathione peroxidase (Mkoji et al. 1988a) were suggested to protect the parasite, *S. mansoni* from the mammalian host's respiratory burst, thus enabling the parasite to survive in the host cell. Acid phosphatase in *L. donavani* (Remaley et al. 1984) and Rickettsiales-like organisms in *P. maximus* (Le Gall et al. 1991) reduces the superoxide production by the phagocytic cells. Hervio et al. (1991) suggested a similar protective role for acid phosphatase present in *Bonamia ostreae* against the host's (*O. edulis*) defense.

Preliminary studies in our laboratory using standard assay procedures failed to detect superoxide dismutase, catalase and glutathione peroxidase in *P. marinus* meronts (unpublished results). However, relatively high concentrations of intracellular (94 mU/mg protein) and extracellular (42 mU/10^6 cells, unpublished results) acid phosphatase activity were observed in *P. marinus* meronts compared to host hemocytes and serum. Acid phosphatase activities in whole hemolymph, supernatant and hemocyte pellets in *C. virginica* were 1.8, 0.7, and 47 mU/mg protein and in *Mercenaria mercenaria*, 3.5, 1.5, and 10.3 mU/mg protein, respectively (Cheng and Rodrick 1975). Our recent studies (Chapter 4) also revealed that growth rate of *P.*

marinus is higher at higher temperatures (upto 25°C). The concentration of extracellular acid phosphatase in the culture medium was positively correlated with cell number and temperature (see Chapter 4). Sodium-L-tartrate is known to inhibit certain acid phosphatases (Le Gall et al. 1991). While acid phosphatases from most isolates of *Leishmania spp.* were inhibited by 2mM L(+)tartrate (77 to 97% inhibition), acid phosphatases from *Leishmania major* isolates were quite tartrate - resistant (7.2% inhibition at 2mM) (Lovelace and Gottlieb 1986). The superoxide ion suppression by sodium-L-tartrate (5 µm/ml) incubated *P. marinus* (for 2 hours) was low compared to live *P. marinus*, however, the differences were insignificant (results not shown). This may suggest that the acid phosphatase(s) in *P. marinus* may be tartrate resistant. The possible deficiency of detectable levels of other anti-oxidant enzymes, and presence of acid phosphatase in *P. marinus* meronts, suggest that acid phosphatase in *P. marinus* may be one of the enzymes which suppresses the ROI production by the host's hemocytes. Acid phosphatase isolated from the cell membrane of *L. donavani* promastigotes catalyzes the dephosphorylation of phosphoproteins including pyruvate kinase, phosphorylase kinase, and histones (Remaley et al. 1984). Phagocytic-cell activation and associated ROI production is regulated by phosphorylation and dephosphorylation of membrane proteins (Andrews and Baboir 1983). Increased *P. marinus* cells in oysters at higher

temperatures may lead to increased acid phosphatase secretion in nature, and may aid the parasite in suppressing the ROI production, possibly by dephosphorylating the enzymes responsible for ROI production eg. Protein kinase C, NADPH oxidase etc. By suppressing the ROI production, *P. marinus* may thus be escaping one of the most important defense mechanism of the host. However, increased CL in infected oysters compared to uninfected oysters was observed by Anderson et al (1992a). This may be due to parasite induced tissue damage and thus a pathological effect and not a defense function.

The exact mechanism of the suppression of ROI production of oyster hemocytes by *P. marinus* meronts is not known at this time and needs further examination.

CHAPTER 5

A COMPARATIVE STUDY OF ACID PHOSPHATASE ACTIVITY IN THE PROTISTAN PARASITE, *PERKINSUS MARINUS* AND ITS' HOST, *CRASSOSTREA VIRGINICA*.

ABSTRACT

Acid phosphatase (AP) in parasites, has been postulated to play a role in compromising the host defense through dephosphorylation of host phosphoproteins and/or inhibition of the oxygen intermediates released by the host phagocytes. Intracellular AP activity in two life stages of the oyster parasite, *Perkinsus marinus*, namely meronts and freshly isolated prezoosporangia were compared. Activity in hemocytes and serum of the host, *Crassostrea virginica*, from different geographical regions were also compared. In addition, the effect of temperature (4, 12, 20 and 28°C) and osmolality (400, 570 and 840 mOsm/kg) on extracellular AP secretion *in vitro* into the culture medium were also investigated. Hemocytes of oysters from James River, Virginia, showed significantly ($p < 0.05$) higher AP activity than those from Damarsicotta River, Maine. AP activity in hemocytes as well as *P. marinus* cell stages increased with temperature ($p < 0.05$). Meronts had significantly higher AP activity than prezoosporangia ($p < 0.0001$). The extracellular AP secretion by *P. marinus* was dose-dependent ($p < 0.001$). Temperature significantly affected AP secretion by *P. marinus* ($p < 0.0001$). Increasing temperatures resulted in increased growth and AP secretion by *P. marinus*. Similarly, osmolality significantly affected AP secretion by *P. marinus* ($p < 0.0001$). Increased growth was seen in meronts in 570 mOsm/kg

media. However, AP activity/10^6 cells was higher for meronts in 400 and 840 mOsm/kg media. Acid phosphatase may play a role in parasites' nutrition and avoidance of host defense.

INTRODUCTION

The protistan parasite, *Perkinsus marinus* has been responsible for severe mortalities of oysters on the eastern coast of the United States and southern Chesapeake Bay (Ragone-Calvo and Burreson 1995). *P. marinus* is a histozoic parasite, however, it is also found in hemocytes of oysters. *P. marinus* infection in oysters increases with increases in temperature and salinity (Andrews 1988).

Host defense in both vertebrates and invertebrates involves various humoral and cellular factors. Molluscs, despite lacking specific immune response and immunoglobulins like their vertebrate counterparts, possess a very effective defense system comprising of both humoral and cellular components (Feng 1988, Chu 1988). Destruction of the parasite by extra- and intracellular lysosomal enzymes, agglutinins, and hemolysins constitute the humoral defense factors. Cellular factors include encapsulation and phagocytosis and form the primary line of defense in molluscs (Fisher 1988, Feng 1988).

Phagocyte membranes when stimulated by foreign particles or soluble products, engage in a process called "oxidative burst". This process involves production of toxic oxygen radicals (reactive oxygen intermediates, ROI) such as OH^-,

H_2O_2, $^1O_2$, and O_2^- which are believed to be microbicidal (Nathan et al. 1979, Murray et al. 1979, Adema et al. 1991). To protect their cell membranes from self damage by lipid peroxidation, the host organisms posses antioxidant enzymes (superoxide dismutase (SOD), catalase, cytochrome C peroxidase, glutathione peroxidase etc). Similar to vertebrate phagocytes, oyster hemocytes showed respiratory burst phenomenon when stimulated by zymosan. Luminol enhanced chemiluminescence has been used to measure respiratory burst activity of oyster hemocytes (Fisher et al. 1990, Anderson et al. 1992).

In a host parasite relationship, the survival of the parasite depends on its ability to evade the defense of the host organism and acquire nutrients for development and proliferation. It has been shown that protozoan parasites, (e.g. *Leishmania*, *Toxoplasma*, and *Trypanosoma* spp.) have the capacity to be phagocytosed without stimulating a respiratory burst and escape the superoxide dependent killing by secreting antioxidant enzymes such as catalase, superoxide dismutase (Weiss et al. 1987) cytochrome C peroxidase, glutathione peroxidase and glutathione reductase (Mkoji et al. 1988a, 1988b) and acid phosphatase (Remaley et al. 1984, Le Gall et al. 1991). In addition, due to the wide substrate specificity of acid phosphatase, although its physiological role in the parasite is not known, acid phosphatase is believed to be

involved in obtaining nutrition from the host (Gottlieb and Dwyer 1981, Glew et al 1982).

Only limited degradation of phagocytosed *P. marinus* meronts in oyster hemocytes was noted in earlier studies (La Peyre 1993, Bushek 1994). Hemocytes challenged with *P. marinus* meronts did not elicit any CL response (La Peyre 1994, Volety and Chu, 1995). These results suggest that the oyster lysosomal enzymes may not be effective in degrading *P. marinus*, or the parasite may posses an effective mechanism(s) to evade the host defense. Results from Chapter 2 (Volety and Chu, 1995) indicate that *P. marinus* is able to suppress hemocyte CL in oysters. Preliminary studies indicate that other antioxidant enzymes (SOD, catalase, glutathione peroxidase) were not detected, but, acid phosphatase was detected in higher levels than in host. Since temperature and salinity were important in regulating the interaction of oyster-*P. marinus*, this study was conducted to examine the effects of temperature and salinity (osmolality) on acid phosphatase secretion by *P. marinus*. Acid phosphatase activities the host *C. virginica* hemocytes as well as plasma were also investigated.

MATERIALS AND METHODS

Hemolymph collection:

Oyster hemolymph was withdrawn from the anterior adductor muscle of oysters through a notch, using a syringe fitted with a 27 gauge needle. Hemocytes and hemolymph were separated by centrifugation (50 x g for 8 min at 5°C).

P. marinus cells:

P. marinus meronts were cultured according to Gauthier and Vasta (1993) using modified DMEM:HAM's F-12 medium. Cells in the log growth phase were used for the experiments. For dose experiments, meronts were resuspended in culture medium at concentrations described below. Prezoosporangia were isolated from infected oyster tissue according to Chu and Greene (1989).

Protein Concentration:

Protein concentration of hemocyte, meront and prezoosporangia lysate was assayed according to Lowry et al. (1951). Bovine serum albumin was used as a standard.

Acid Phosphatase Activity:

Acid phosphatase activities in hemocyte, meront and prezoosporangia lysate and culture medium were assayed using a colorimetric assay (Sigma Diagnostics) based on the release

of p-nitrophenol and inorganic phosphate from p-nitrophenyl phosphate by the enzyme (Andersch and Szczypinski 1947). Results were expressed as specific activity (units/mg protein), units/10^6 cells or units/ ml of culture medium. One unit is described as the amount of enzyme activity that will liberate 1 µM of p-nitrophenol per hour at 37°C.

Cell Growth:

P. marinus cell growth was determined using a MTT-based Cell Growth Determination kit (Sigma Diagnostics). Standard curves were constructed using cells cultured at appropriate osmolality media (see below) for the salinity experiment.

Experiments:

1. Comparison of intra- and extra-cellular acid phosphatase activity in oysters:

Hemocytes and hemolymph serum were used to determine the intra- and extra-cellular acid phosphatase activities in oysters. Oysters obtained from the Deep Water Shoal area, James River, Virginia, and Damarsicotta River, Maine were divided into three groups. Hemolymph samples from several oysters from each group were withdrawn and pooled to yield sufficient numbers of hemocytes for the acid phosphatase assay. Hemocyte and serum fractions were separated into different tubes and hemocytes were thoroughly washed free of

serum using 1 µm filtered estuarine water (salinity = 20 ppt). Hemocytes were then resuspended in water containing 0.1% Triton X-100, and sonicated for three minutes using a Heat Systems, Ultrasonic sonicator Model W-10. The cell lysate was then centrifuged at 20,000 x g for 10 min and the supernatants were collected. Triplicate samples were analyzed. AP activity in hemocyte lysate and hemolymph serum were assayed at 10, 15 and 25°C.

2. Comparison of intra-cellular acid phosphatase activity in meronts and prezoosporangia:

P. marinus meronts and prezoosporangia were washed free of culture medium and tissue homogenate respectively, using 1 µm filtered estuarine water (20 ppt) and resuspended in water with 0.01% Triton X-100. Both meronts and prezoosporangia (N=4) were sonicated for three minutes as described above, centrifuged at 20,000 x g for 10 min and supernatants collected. AP activity was then determined in *P. marinus* cell lysate at 10, 15 and 25°C.

3. Dose response of *P. marinus* on AP secretion in vitro:

P. marinus meronts were resuspended in culture medium at a concentration of 0, 1, 2, 4 and 8 x 10^6 cells/ml. Two ml of culture medium with the above mentioned concentration of meronts (N=6) were incubated in 24 well culture plates at 28°C

for a period of 48 hours. At the end of the incubation period, cells and media were separated by centrifugation. AP activities in the culture media were then determined.

4. Effect of temperature on AP secretion by *P. marinus*:
P. marinus meronts at a concentration of 4 x 10^6 cells/ml were placed in 24 well culture plates (N=6) and incubated at 4, 12, 20 and 28°C for a period of 48 hours. After incubation, cell growth (in millions of cells) was determined. Cells were separated from the culture medium and AP activity was analyzed. AP activity was expressed as units/10^6 cells.

5. Effect of osmolality on AP secretion by *P. marinus*:
Similar to the temperature experiment, a cell concentration of 4 x 10^6 cells was used in salinity effect experiments. Osmolality of the culture media was adjusted to 400, 570 and 840 mOsm/kg (equivalent to ~14, 20 and 28 ppt respectively) using synthetic salt (Aquarium Systems, Mentor, Ohio). Meronts were resuspended in media of different osmolality in 24 well culture plates and incubated at 28°C for a period of 48 hours. After incubation, cell growth and AP activity in culture media were determined using procedures described above. Results were expressed as units/10^6 cells.

STATISTICAL ANALYSES

A Two-Way analysis of variance (ANOVA) was used to determine the difference in acid phosphatase activity between hemocytes and serum in oysters from two locations; and between meronts and prezoosporangia at different temperatures. A One-Way ANOVA was used to determine the differences in AP activity: i) between doses of *P. marinus* incubated media; ii) in media with *P. marinus* incubated at different temperatures; and iii) *P. marinus* incubated in different osmolarity media. Regression and correlation analyses were also used to determine the relation between cell number and acid phosphatase concentration in *P. marinus* culture medium in both temperature and salinity effect experiments.

RESULTS

Significant differences in acid phosphatase activity in hemocytes from James River and Damarsicotta River oysters were observed ($p < 0.05$). Acid phosphatase activity was higher in hemocytes from James River oysters than Damarsicotta River oysters at all temperatures (Fig 1). Acid phosphatase activity in hemocytes from oysters from both the locations was higher at 25°C compared to 10 and 15°C ($p < 0.05$). However, acid phosphatase activity was below detection limits in serum of oysters from both the locations. Results also indicate

that acid phosphatase activity in hemocytes from oysters from both locations, increases with increasing temperature (Fig 1) ($p < 0.05$).

Meronts had significantly higher acid phosphatase activity compared to prezoosporangia ($p < 0.0001$) at all temperatures examined in the present study (Fig 2). Similar to hemocyte acid phosphatase, *P. marinus* acid phosphatase activity also increased with an increase in temperature. Acid phosphatase activity in *P. marinus* cells was significantly higher at 15 and 25°C, compared to 10°C ($p < 0.01$).

Dose dependent acid phosphatase activity was observed in *P. marinus* culture media after 48 hours of incubation (Fig 3). While no acid phosphatase activity was observed in the control (meront-free media), significantly higher activity was seen in culture medium incubated with 2, 4 or 8 x 10^6 cells/ml, compared to medium with 1 x 10^6 cells/ml ($p < 0.001$). When acid phosphatase activity was expressed as units/10^6 cells, no significant differences ($p > 0.05$) were observed in culture medium seeded with different doses of meronts, indicating that acid phosphatase secretion into culture medium was dose related.

Acid phosphatase activities in culture medium with *P. marinus* incubated at different temperatures are shown in Fig

Fig 4. Acid phosphatase activity significantly increased with increase in temperature ($p < 0.0001$). The increase of acid phosphatase activity in culture medium increased with an increase in meront cell number at the end of 48 hours of incubation. Acid phosphatase activity (units/10^6 cells) was significantly higher in culture media with meronts at 28 and 20°C than at 4 and 12°C (Fig 4). In addition, a strong correlation existed (0.977) between number of meront cells at all temperatures and acid phosphatase activity in the culture medium (Fig 5).

Acid phosphatase concentration was higher in culture medium at 570 mOsm/kg than 400 and 840 mOsm/kg ($p < 0.0001$). Similar to acid phosphatase concentration, cell number of *P. marinus* after 48 hours of incubation also significantly increased in culture media at 570 mOsm/kg compared to 400 and 840 mOsm/kg. A strong correlation (0.885) was observed between meront cell number in media at all osmolarities and acid phosphatase media concentration (Fig 6). However, when results were expressed as acid phosphatase activity/10^6 cells, meronts in media at 400 and 840 mOsm/kg had higher acid phosphatase activity compared to ones at 570 mOsm/kg ($p < 0.0001$) (Fig 7).

Fig 1: Mean acid phosphatase activity (units/mg protein ± SE) in hemocytes of oysters from the James River, Virginia, and Damarsicotta River, Maine assayed at 10, 15 and 25°C. CBAY = James River oysters, MAINE = Damarsicotta River oysters.

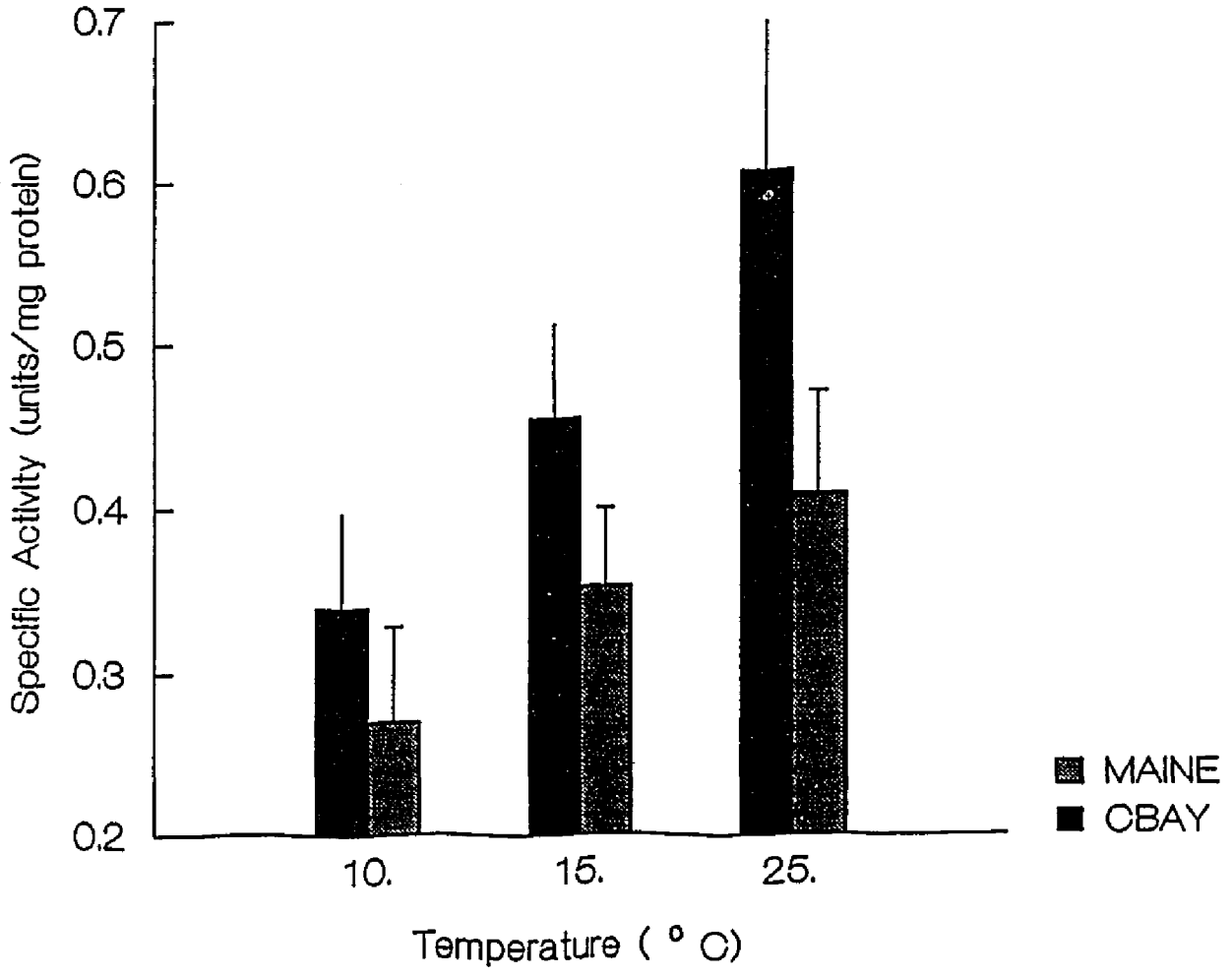

0.7
0.6
0.5
0.4
0.3
0.2
Specific Activity (units/mg protein)
10.
15.
25.
Temperature (° C)
MAINE
CBAY

Fig 2: Mean acid phosphatase activity (units/mg protein ± SE) in cultured meronts and prezoosporangia isolated from infected oyster tissue assayed at 10, 15 and 25°C. HYPNOSP = hypnospores (prezoosporangia), MERONT = cultured meronts.

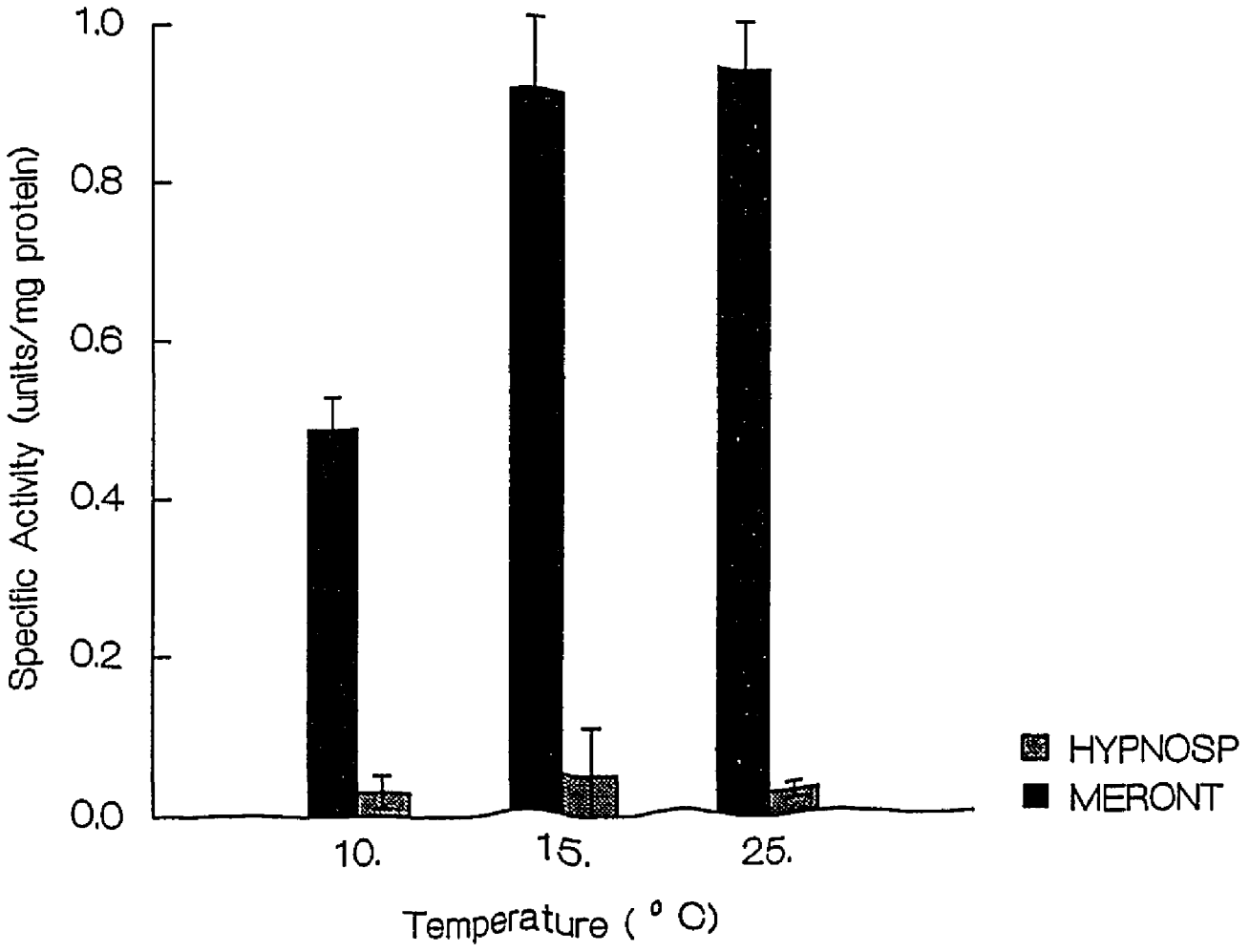

1.0
0.8
0.6
0.4
0.2
0.0
Specific Activity (units/mg protein)
10.
15.
25.
Temperature (° C)
HYPNOSP
MERONT

Fig 3: Mean acid phosphatase activity (units/ml ± SE) in culture medium with initial density of 0, 1, 2, 4, and 8 x 10^6 meronts/ml after 48 hrs of incubation. bars with dissimilar letters denotes significance ($p < 0.05$).

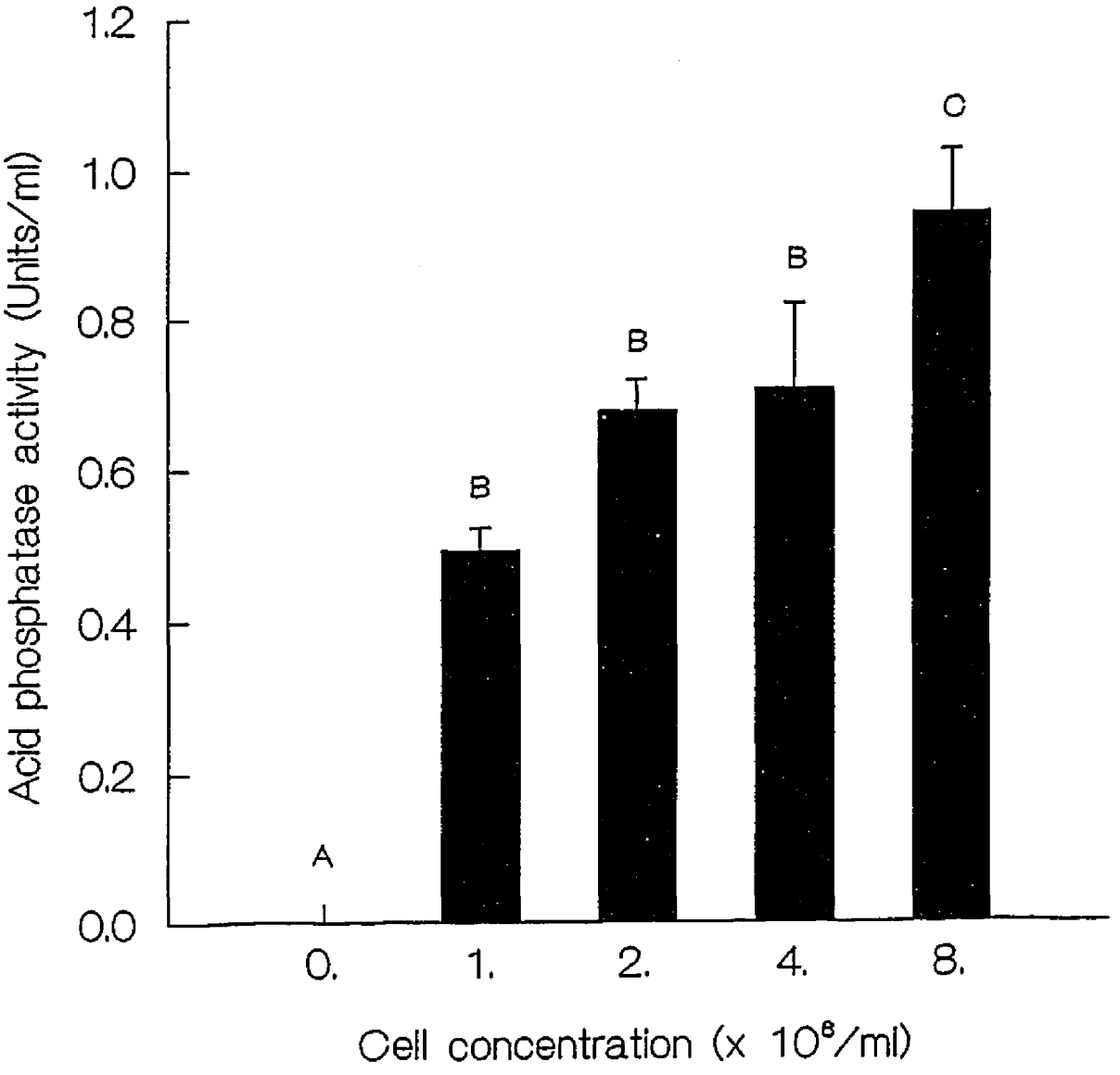

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Acid phosphatase activity (Units/ml)
A
B
B
B
C
0.
1.
2.
4.
8.
Cell concentration (x 10⁶/ml)

Fig 4: Mean acid phosphatase activity (units/10^6 cells ± SE) in meront containing culture medium incubated at 4, 12, 20 and 28°C after 48 hrs of incubation.

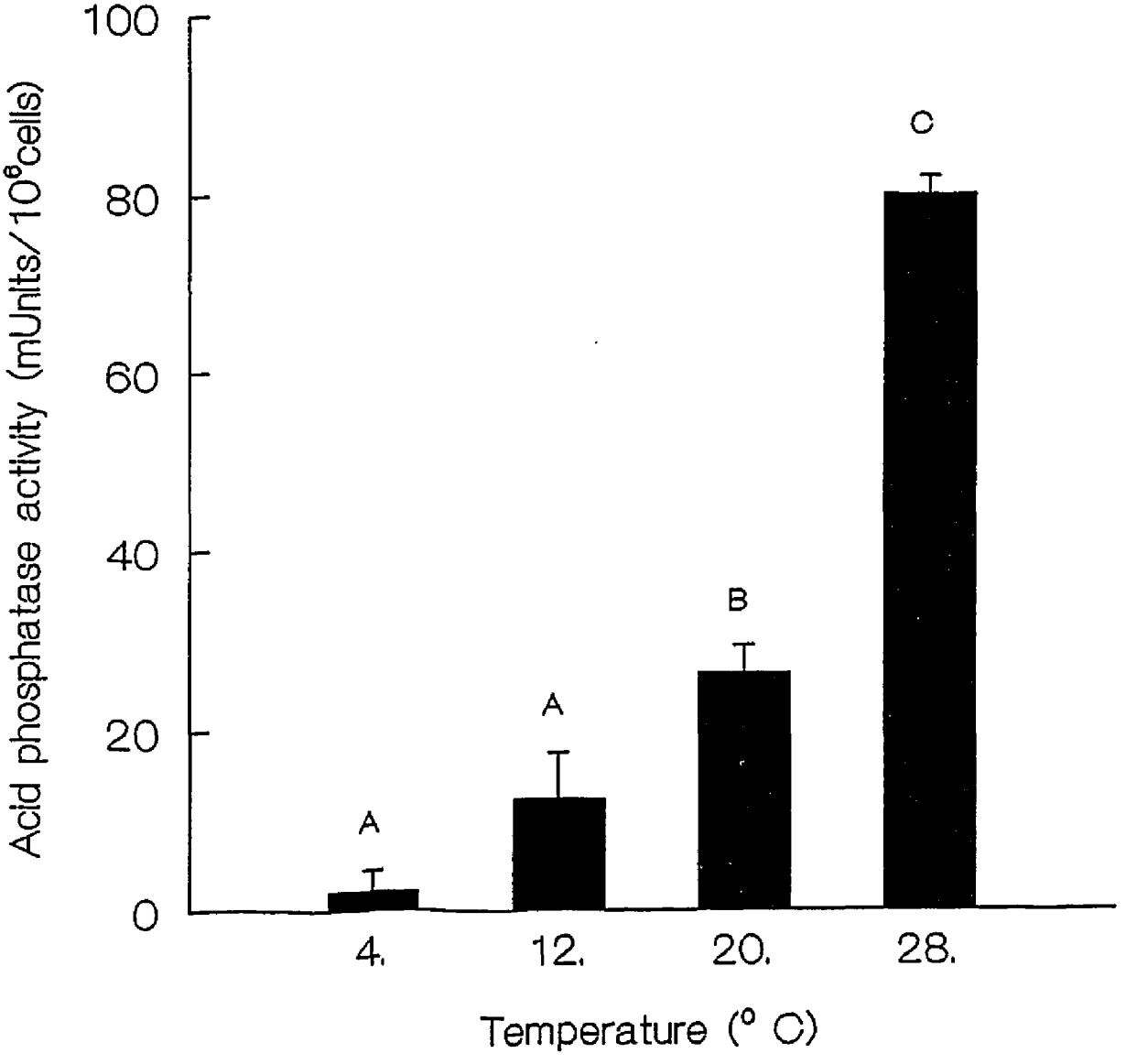

100
80
60
40
20
0
Acid phosphatase activity (mUnits/10^6cells)
A
A
B
C
4.
12.
20.
28.
Temperature (° C)

Fig 5: Mean acid phosphatase concentration in culture medium (units/ml) after 48 hrs of incubation at 4, 12, 20 and 28°C. Corr = Correlation coefficient. Regression equation is denoted at the top of the graph.

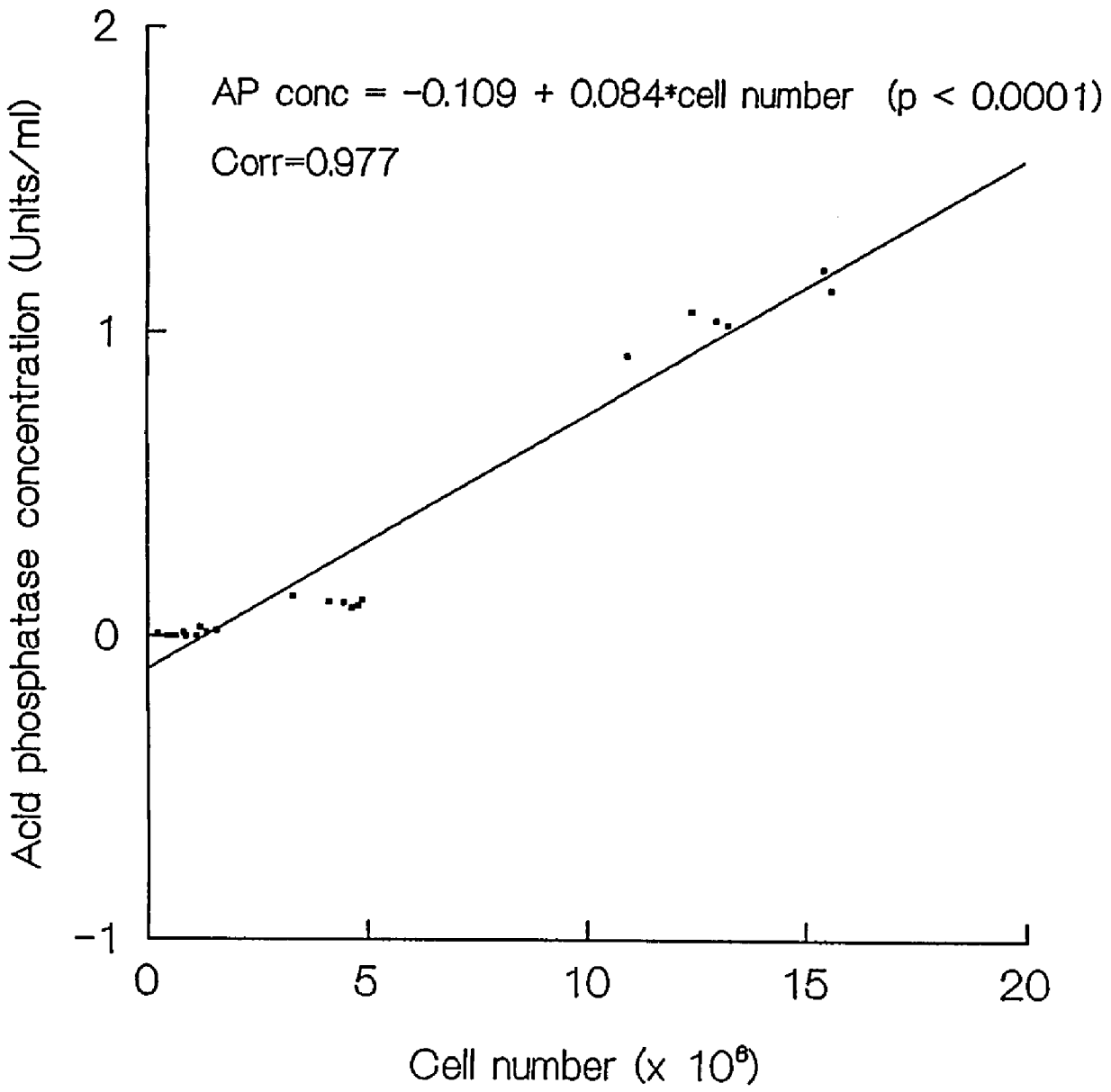

AP conc = −0.109 + 0.084*cell number (p < 0.0001)
Corr=0.977
Acid phosphatase concentration (Units/ml)
2
1
0
−1
0
5
10
15
20
Cell number (x 10⁶)

Fig 6: Mean acid phosphatase concentration (units/ml) in culture medium with an osmolality of 400, 570, and 840 mOsm/kg after 48 hrs of incubation at 28°C.

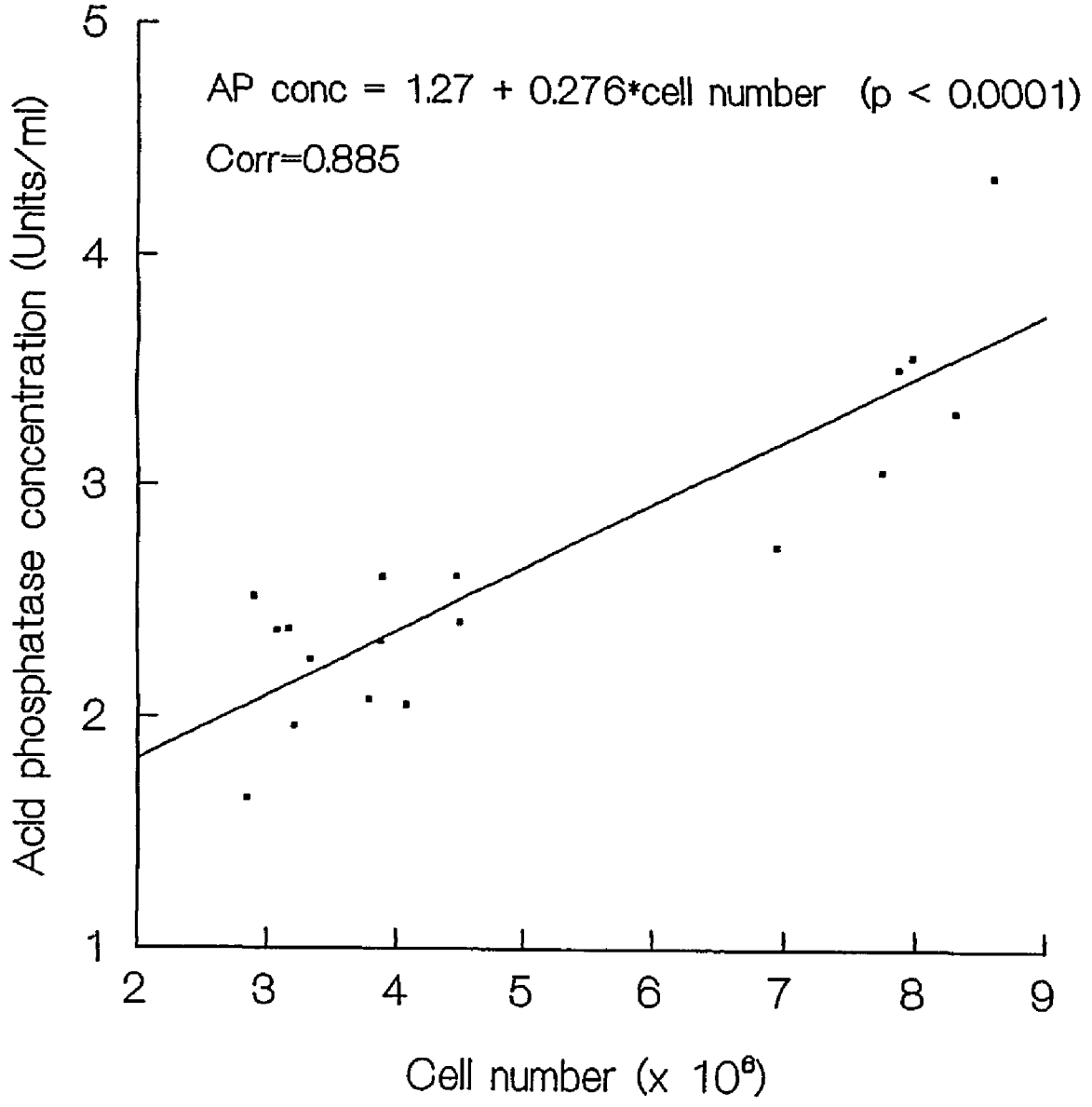
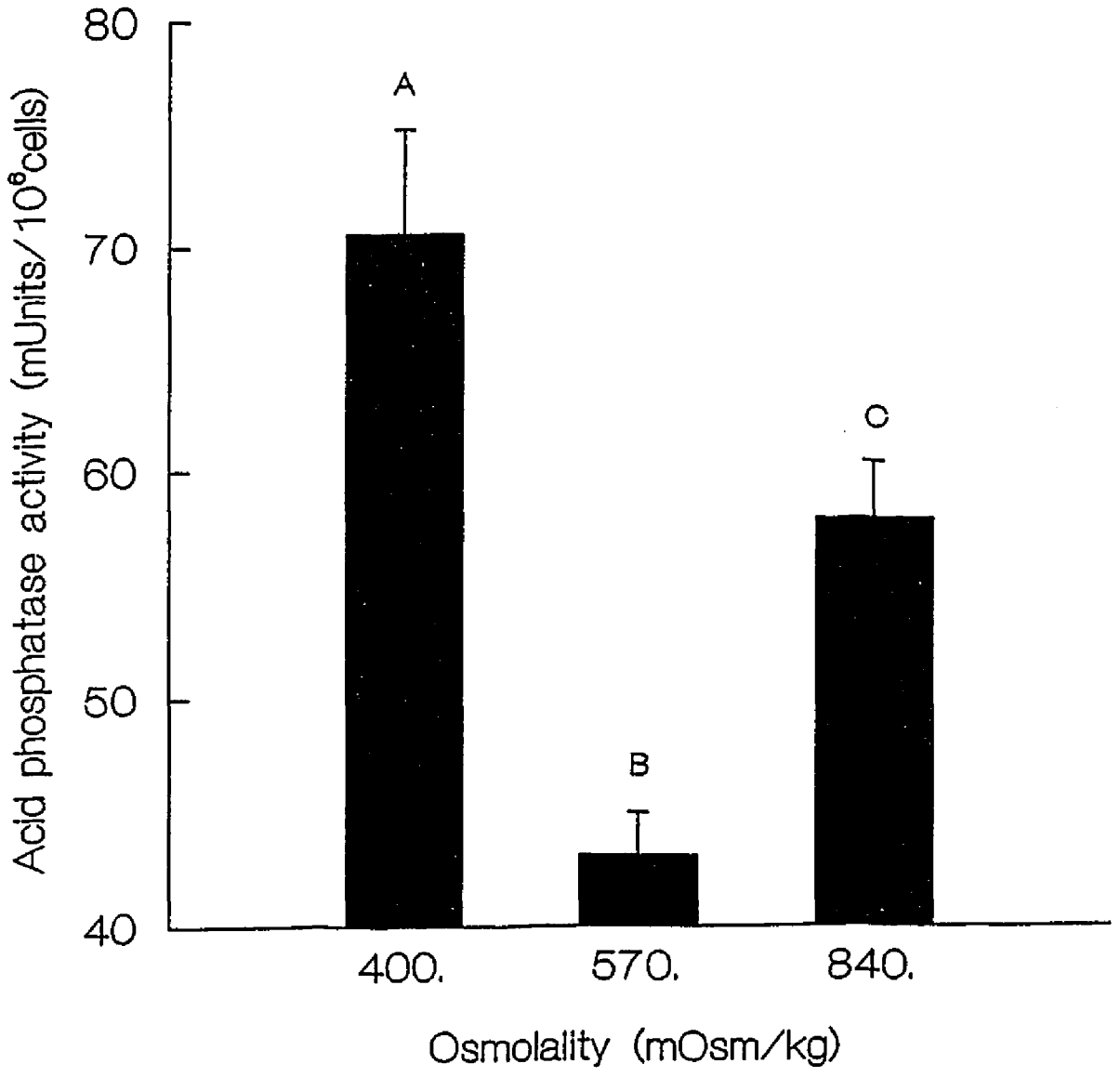

AP conc = 1.27 + 0.276*cell number (p < 0.0001)
Corr=0.885
Acid phosphatase concentration (Units/ml)
Cell number (x 10⁶)
1
2
3
4
5
2
3
4
5
6
7
8
9

Fig 7: Mean acid phosphatase activity (units/10^6 cells ± SE) in meront containing culture medium with osmolality of 400, 570, and 840 mOsm/kg at 28°C after 48 hrs of incubation.


80
70
60
50
40
A
B
C
400.
570.
840.
Acid phosphatase activity (mUnits/10⁶cells)
Osmolality (mOsm/kg)

DISCUSSION

Results indicate a significant difference in acid phosphatase activity in hemocytes of oysters from two different populations of oysters, Chesapeake Bay and Maine. The difference in acid phosphatase activity in hemocytes may be related to the differences in habitat, environmental history or genetic factors.

Although, acid phosphatase is generally considered as a "lysosomal marker", it has been found to be membrane bound in some mammalian cells (Solyom and Trams 1972) and some microorganisms (Aaronson 1973, Eeckhout 1973) including trypanosomatids (Gottlieb and Dwyer 1981). acid phosphatase in *Leishmania* has been hypothesized to play an important role in nutrition of the parasite by dephosphorylating host phosphoproteins and/or phosphorylated amino acids (Lovelace et al. 1986, Lovelace and Gottlieb 1986).

Results from the present study demonstrate that acid phosphatase activity in *P. marinus* meronts is higher than oyster hemocytes and serum. The high acid phosphatase activity in *P. marinus* may be responsible for the suppression of hemocyte CL (Volety and Chu 1995). Acid phosphatase may dephosphorylate enzymes responsible for the production of ROI by oyster hemocytes. Oysters infected with *P. marinus* (with

an intensity of moderate to heavy) have a decreased condition index compared to lightly infected or uninfected oysters (Paynter and Burreson 1991, Dittman 1993, Volety and Chu 1994). The dose dependent response between *P. marinus* meronts and acid phosphatase activity in the culture medium (Fig 3) may suggest that increasing Dermo infection as a result of high proliferation of parasite results in increased acid phosphatase activity which may break down the host phosphoproteins and may serve the function of nutrition thus possibly leading to reduced condition index of oysters.

Both laboratory and field studies indicate that *P. marinus* infection in oysters increases with increasing temperature (Chu and La peyre 1993, Andrews 1988, Soniat 1985). The possible correlation (Fig 5, Corr 0.977) between cell number and acid phosphatase concentration in culture medium suggests that cell growth rate is higher at elevated temperatures which results in increased in acid phosphatase concentration. Temperatures above 28°C are inhibitory to the growth of *P. marinus* (Volety and Chu, unpublished results). Increased acid phosphatase activity/10^6 cells with increasing temperatures (Fig 4) suggests that temperature significantly affects not only the growth rate of *P. marinus*, but also, the acid phosphatase secretion activity of the parasite. The pattern of increased growth rate of *P. marinus* and acid phosphatase secretion activity at higher temperatures *in*

vitro, is similar to the pattern of increased Dermo infections at higher temperatures observed in the field and laboratory studies. The highest disease prevalence and associated mortalities of oysters occur during mid to late summer when temperatures are above 25°C (Ray 1954, Andrews and Hewatt 1957, Andrews 1988). Increasing temperatures perhaps increase the metabolic functions, and thus the growth rate and acid phosphatase secretion by the parasite. Increased acid phosphatase secretion at higher temperatures (> 20°C) may dramatically result in dephosphorylation of phosphoproteins and/or phosphorylated amino acids of the host for nutrition.

Results of osmolality (salinity) effect suggest that 570 mOsm/kg (20 ppt) is an optimal osmolality (salinity) for *P. marinus* growth. The osmolarity of 840 mOsm/kg (28 ppt) or 400 mOsm/kg (14 ppt) may be stressful for the growth of the parasite. Laboratory studies by Burreson et al. (1994) revealed that heavy mortalities occurred in cultured *P. marinus* cells incubated in estuarine water of osmolality 290 mOsm (~9ppt) for 24 hrs. Field (Soniat 1985, Soniat and Gauthier 1989, Craig et al. 1989, Paynter and Burreson, 1991), and laboratory studies (Chu et al. 1993, Ragone and Burreson 1993) have documented that Dermo infection in oysters is low at lower salinities. Low salinities effect the physiology of the oysters and parasite, thus influencing the outcome of the disease (Scott et al. 1985). Although, media at 570 mOsm

supported highest growth rate and acid phosphatase concentration, when results were expressed as acid phosphatase activity/10^6 cells, it had significantly lower activity/cell compared to the other two osmolarities (salinities) examined. Possibly, when the growth of *P. marinus* is slowed at both very low and high osmolalities (salinities), they may be using their energy in producing high levels of acid phosphatase. However, the exact reasons for the decreased acid phosphatase secretion by *P. marinus* in the culture medium which sustains maximum growth is difficult to explain at the present time. Further studies are necessary to investigate this result.

Apart from nutrition and suppression of ROI production, acid phosphatase may also be involved in other defense functions for the parasite. Acid phosphatase has been implicated in the virulence and infectivity of *L. donavani* (Katakura and Kobayashi 1988). Qualitative and quantitative differences in acid phosphatase activity have been observed between virulent and avirulent *L. donavani* promastigotes in their study. In addition, it has been suggested that phosphotyrosine phosphatases may be responsible for the virulence in the bacteria of genus *Yersinia* which affects humans (Walton and Dixon 1993). The higher acid phosphatase activity in meronts compared to prezoosporangia may be one of the reasons for the higher virulence of meronts. Involvement of phosphatases in signalling interferon γ and signal

transduction (see Walton and Dixon 1993), DNA synthesis (Brautigan 1992), regulation of cell cycle (Freeman and Donoghue 1991) has also been described. Phosphatases and kinases activate and deactivate each other thereby regulating the cell cycle and events thus leading to DNA, RNA and protein synthesis (Meek and Street 1992). Electron microscopic studies investigating localization of acid phosphatase in *P. marinus* have revealed that acid phosphatase activity is mainly localized in the nucleus and partly in the cell membrane (Chapter 6). Thus, acid phosphatase in *P. marinus* may be involved in functions such as nutrition, ROI inhibition and cell cycle regulation.

In summary, increasing temperatures and salinities increase the growth rate of *P. marinus* causing higher infections in oysters at elevated temperatures and salinities. In addition, higher temperatures also increase the metabolic rate of the parasite resulting in higher acid phosphatase secretion/cell. Investigations of Bushek (1994) indicate that differences in virulence exists between different strains of *P. marinus* from different geographical locations. Further studies are necessary to characterize acid phosphatase in *P. marinus*, investigate if any qualitative and quantitative differences exist between *P. marinus* strains, and examine the role of acid phosphatase in parasites' survival and virulence.

CHAPTER 6

ULTRASTRUCTURAL LOCALIZATION OF ACID PHOSPHATASE IN *PERKINSUS MARINUS*, THE APICOMPLEXAN PARASITE OF THE AMERICAN OYSTER, *CRASSOSTREA VIRGINICA*.

ABSTRACT

Distribution of acid phosphatase activity was localized at the ultrastructural level in *Perkinsus marinus*, an apicomplexan parasite which causes heavy mortalities in the American oyster, (*Crassostrea virginica*) populations. Acid phosphatase activity was localized by lead phosphate precipitation, with sodium glycerophosphate as the substrate using electron microscopy. Intense acid phosphatase activity was found in the nucleus, while moderate activity was observed in the plasma membrane. Acid phosphatase activity was inhibited by sodium fluoride. Based on the location and distribution, acid phosphatase may aid the parasite in obtaining nutrients from the host and maybe involved in cell cycle regulation. The role of acid phosphatase in suppressing production of reactive oxygen intermediates' from the host hemocytes, in nutrient requirements and in cell regulation mechanisms are discussed.

INTRODUCTION

In recent years, the apicomplexan protozoan parasite, *Perkinsus marinus*, has caused severe mortalities in the native oyster populations, *Crassostrea virginica*, on the east coast of the United States and southern Chesapeake Bay in particular (Andrews, 1988; Burreson, 1989). The physiopathologic effects of *P. marinus* on its host have been extensively investigated (Paynter and Burreson 1991). Molluscs, despite lacking a well developed immune system, possess a very effective defense system, comprised of cellular (Fisher, 1988; Feng, 1988) and humoral components (Chu, 1988; Cheng and Rodrick, 1975). The humoral component consists of lysosomal enzymes, lectins and agglutinnins; while the cellular system involves phagocytosis and encapsulation by granular and agranular hemocytes. Although, extracellular lysozyme activity has been negatively correlated with *P. marinus* infection in oysters, it has not been proven that lysozyme effectively helps oysters in resisting *P. marinus* infection. Internalization and limited degradation of *P. marinus* cells in oyster hemocytes has been documented in earlier studies (La Peyre, 1993; Bushek et al., 1994). This suggests that the lysosomal enzymes may not be effective in killing the parasite, or alternatively, the parasite may posses mechanisms which enables it to survive in the hostile environment of the host cell. For example, secretion of proteases and proteolytic enzymes (Pino-Hess et

al., 1985), and secretion of antioxidant enzymes by parasites (Weiss et al., 1987) to escape the superoxide-dependent microbicidal activity (Horan et al., 1982; Welch 1980) by inhibiting/suppressing the reactive oxygen intermediates (Remaley et al., 1984; Le Gall et al., 1991) was demonstrated. Investigations in our laboratory indicated that *P. marinus* suppresses/inhibits production of reactive oxygen intermediates by oyster hemocytes (Volety and Chu, In press). It has been reported that such mechanisms exist in other parasites, such as *Leishmania* (Remaley, 1984), *Bonamia ostreae* (Hervio, personal communication), and rickettsiales-like organisms (Le Gall et al., 1991). The enzyme, acid phosphatase, was suggested to be involved in the suppression of the superoxide ion production by the host phagocytes (Remaley et al., 1984). Besides being involved in the suppression of superoxide ion production, acid phosphatase plays a role in nutrition by dephosphorylating host phosphoproteins and/or phosphorylated amino acids (Lovelace et al., 1986; Lovelace and Gottlieb, 1986). Our preliminary studies indicate that *P. marinus* secretes high levels of intra- and extracellular acid phosphatase (Chapter 5). In an attempt to elucidate the role of acid phosphatase in *P. marinus*, we have localized acid phosphatase in cultured *P. marinus* cells using electron microscopy.

MATERIALS AND METHODS

P. marinus culture:

P. marinus cells were cultured according to Gauthier and Vasta (1993). Parasites from log phase of growth were used in this study. *P. marinus* cells were subcultured in 25 cm^2 culture flasks containing 10 mls of modified DMEM:HAMs F-12 medium.

Electron microscopy:

Localization of acid phosphatase was carried out according to the modified procedure of Gomori (1950). Parasite cultures were thoroughly washed with 1 µm filtered sea water (27 ppt) and fixed with cold calcium-paraformaldehyde, pH 3.8 [(anhydrous calcium chloride (1.0g), distilled water (50 ml), 8% paraformaldehyde (50 ml)] for 20 min at 4°C. The cells were then washed with water twice and incubated in the incubation medium containing acetate buffer, pH 5.0 (25ml) (0.6% acetic acid 300ml, 0.2M sodium acetate 700ml), distilled water (100ml), lead nitrate (0.12 g), and 3% sodium glycerophosphate (10ml)) for 4 hrs at 37°C. Control samples were run in parallel. One control was incubated in the medium without the substrate, sodium glycerophosphate and the second control in the test incubation medium with sodium fluoride (0.05M), an acid phosphatase inhibitor. The cells were then washed in water three times and transferred to post fixative (2.5% gluteraldehyde in phosphate buffered saline (PBS) pH

7.0) for 10 min at room temperature. The cells were then washed three times with fresh buffer and transferred to 1% osmium tetroxide in PBS for 10 min at room temperature. After the incubation, the cells were washed several times with fresh buffer and cell pellets dehydrated in a graded series of ethanol (10-100%) and two changes of propylene oxide. The cell pellets were then embeded in Embed 812/Aldrite 502 resin. The pellets were sectioned using a Reichert-Jung Ultracut E microtome, counterstained using Pb/uranyl citrate, and examined using a Jeol 100CX2 electron microscope.

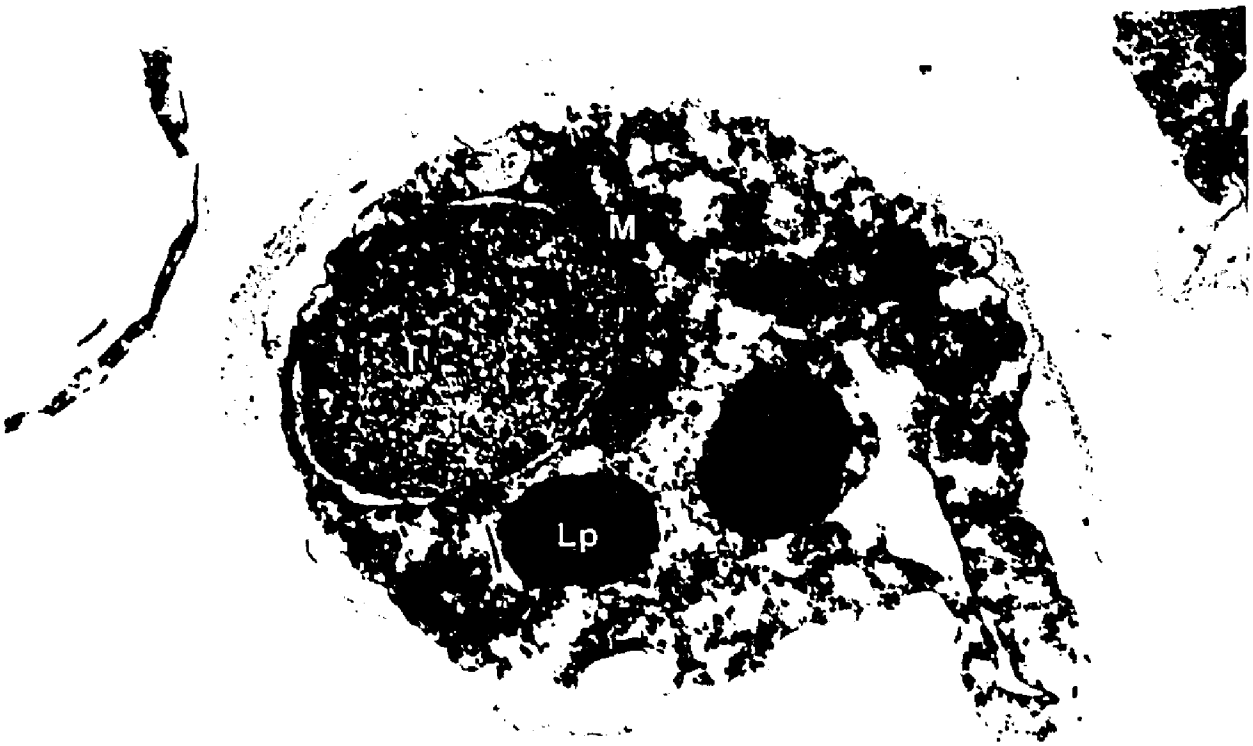
RESULTS

Electron dense deposits of lead phosphate, indicative of acid phosphatase activity were noted in the nucleus of the cells (Fig 1). The acid phosphatase activity was intense in the nucleus. No lead phosphate deposits were observed in any other organelles (for e.g. endoplasmic reticulum, dense bodies, mitochondria). No precipitation of lead nitrate was detected in either control samples (Figs 2 and 3).

Fig 1: Electron micrograph of *P. marinus* showing acid phosphatase localization. *P. marinus* cells were incubated in test medium with the substrate (sodium glycerophosphate). N = Nucleus, V = Vacuoplast, L_p = Lipoid droplet, V_m = Vacuoplast material. Scale bar = 1 µm.

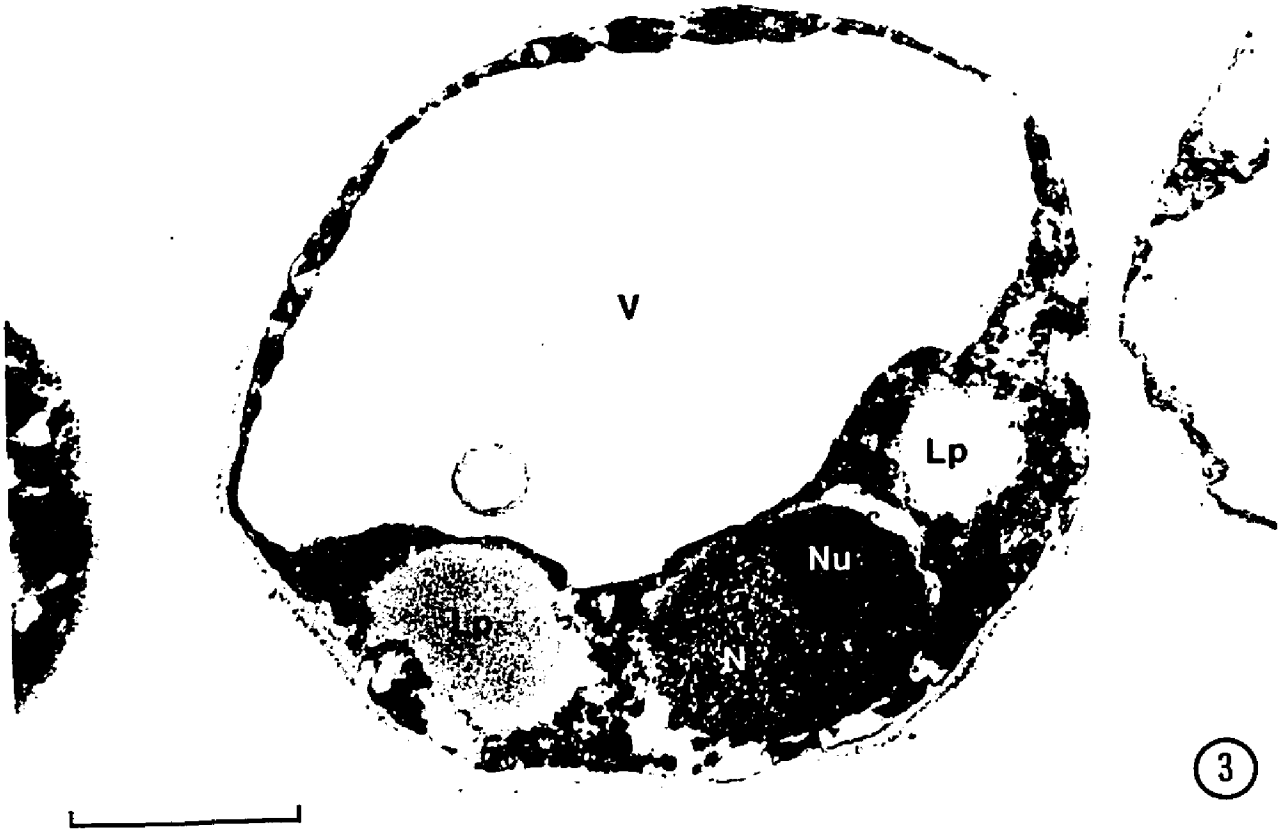

N
N
V
V
Vm
1

Fig 2: Electron micrograph of *P. marinus* incubated in test medium lacking the substrate (sodium glycerophosphate). N = nucleus, M = mitochondria, L_p = Lipoid droplet. Scale bar = 1 µm.



(2)

Fig 3: Electron micrograph of *P. marinus* incubated in test medium with substrate (sodium glycerophosphate) and inhibitor, sodium fluoride. V = vacuoplast, L_p = lipoid droplet, N = nucleus, N_u = nucleolus. Scale bar = 1 μm.



3

DISCUSSION

In general, acid phosphatase has been considered to be a lysosomal-marker. However, presence of acid phosphatase in the cell organelles such as cell membrane in *Leishmania sp.* (Gottlieb and Dwyer, 1981), dense bodies in *Bonamia ostreae* (Hervio et al., 1991), spherites in the larvae of the trematode, *Meigymnophallus minutus* (Azevedo and Corral, 1987), Golgi vesicles, and endoplasmic reticulum of *Thraustochytrium sp.* zoospores (Kazama, 1979) has been reported. Acid phosphatase has been reported in the nucleus in a number of organisms. For example, Sood and khan (1982) reported intense acid phosphatase activity in the nucleus isthmi, nucleus profundus mesencephali and nucleii of cranial nerve cells of the fresh water turtle, *Lissemys punctata granosa*. Acid phosphatase activity was also detected in the nucleii of medullary and cortical cells of the fresh water teleost, *Labeo rohita* (Kulkarni and Satyanesan, 1979) and in the nucleii of the stratified epithelium in the buccopharynx and columnar cells of intestinal bulb, intestine and rectum of the teleost, *Cirrhinus mrigala* (Sinha, 1979). Involvement of phosphatases in signaling interferon γ and signal transduction (see Walton and Dixon, 1993), DNA synthesis (Brautigan 1992), regulating the cell cycle (Freeman and Donoghue, 1991) has also been described. Phosphatases and kinases activate and deactivate each other thereby regulating the cell cycle and events thus

leading to DNA, RNA and protein syntheis (Meek and Street, 1992). Todate, there has been no studies on the localization and distribution of acid phosphatase in the nucleus of an apicomplexan parasite.

The activity of acid phosphatase in *P. marinus* cell membranes seem to resemble those in some other protists. Gottlieb and Dwyer (1981a, 1981b) reported the localization of acid phosphatase in the plasma membrane of *Leishmania donavani*. They speculated that acid phosphatase may play a role in acquiring nutrients from the organic phosphates of host cells and possibly protect the parasite from being digested in either the alimentary tract of the host (sand fly), or in the macrophages of the mammalian host. Acid phosphatase has been found in "dense bodies" of *Bonamia ostreae*, a protozoan parasite of the flat oyster, *Ostrea edulis* (Hervio et al., 1991). Similar to most *Leishmania spp.*, acid phosphatase in *P. marinus* appeared to be highly sensitive to sodium fluoride (Fig 3). Acid phosphatase from *Leishmania spp.* and Rickettsiales-like organisms inhibits superoxide production from host hemocytes (Remaley et al., 1984; Le Gall, 1991).

Production of reactive oxygen intermediates by bovine neutrophils was found to be correlated with the phosphorylation of cytosolic polypetides. This suggests the

involvement of kinases in the processes of superoxide anion production (Gennaro et. al., 1985). They also demonstrated the inhibition of O_2^- production by phorbol myristate acetate stimulated bovine neutrophils when an inhibitor of protein kinase C was used. The relatively high concentration of acid phosphatase detected in the extracellular secretion and the ability to suppress reactive oxygen intermediates production in oyster hemocytes by *P. marinus* secretions suggests that acid phosphatase may act as an antioxidant enzyme by dephosphorylating the enzymes (e.g. protein kinase C and NADPH oxidase) involved in ROI production, thus aiding the parasite to escape the superoxide dependent microbicidal activity. Using standard assay procedures, we did not detect the antioxidant enzymes, catalase, superoxide dismutase, and glutathione peroxidase in *P. marinus*. Also, the secretion of this enzyme into the culture medium suggests a nutritional role for the enzyme. Acid phosphatase in *P. marinus*, in addition to nutrient acquisition, may aid the parasite to avoid or interfere with the hosts' defense. However, further study is needed to determine the role of acid phosphatase in *P. marinus*.

CHAPTER 7

BIOCHEMICAL CHARACTERIZATION OF THE OYSTER, *CRASSOSTREA VIRGINICA* PARASITE, *PERKINSUS MARINUS*: LIPID AND FATTY ACID COMPOSITION.

ABSTRACT

Both meront and prezoosporangia stages of the oyster, parasite, *P. marinus* are characterized by an abundance of refractile bodies which are lipid droplets. To determine the role of lipids and fatty acids in the parasites' development and defense against the host, the lipid and fatty acid composition of meront and prezoosporangia during development were determined. Lipid classes were different in meronts cultured in two different media. Triacylglycerols (TAGs) were the dominant lipid class in meronts cultured in Medium 1, while phospholipids (PLs) were the major lipid class in meronts cultured in Medium 2. No significant differences in total lipid content (µg lipid/mg tissue) indicated that were observed between cultured meronts and prezoosporangia isolated from infected oyster tissue, preincubated in thioglycollate medium. However, the lipid class composition of these two life stages was different. PLs were the major lipid class in cultured meronts, while TAGs were the major lipid class in prezoosporangia. The lipids in meront culture media were primarily wax/cholesterol esters and PLs, while PLs were abundant in thioglycollate medium. Results also indicate that meronts and prezoosporangia have much higher levels of arachidonic acid (20:4n-6) (> 7-11% of the total fatty acids) compared to the oyster (< 2%). This suggests that *P. marinus* may actively assimilate arachidonic acid and/or modify short

chain fatty acids of the n-6 family from the host.

INTRODUCTION

Perkinsus marinus (Dermo) has become the predominant pathogen since the mid-1980s, replacing *Haplosporidium nelsoni* (MSX) as the most important pathogen of the oyster, *Crassostrea virginica*, in the southern Chesapeake Bay and the eastern coast of the United States (Andrews 1988). Although numerous studies have examined host-parasite interactions and physiopathological effects of *P. marinus* on its host (Paynter and Burreson 1991, La Peyre 1993, Volety and Chu 1994), very few studies have investigated the biochemical composition of *P. marinus* in relation to host-parasite interaction.

In a host-parasite interaction, parasites need to obtain nutrients from the host to grow and multiply. The lipid and fatty acid differences between hosts and parasites have been investigated in numerous organisms, e.g., molluscs and the helminth parasite, *Echinostoma capri* (Fried et al. 1989); in mammals and the cestode parasite, *Hymenolepis diminuta* (Jacobsen and Fairbairn 1967); and in mammals and the protozoan parasites of the genus, *Plasmodium* (Holz 1977, Sherman 1979, Vial et al. 1982). Few studies have reported the lipid class and fatty acid composition of both the host and the parasite. The fatty acid composition of *Spirometra erinacei* (Fukushima et al. 1988) closely resembled the composition of its' host mammalian host. Plasma fatty acids

and lysophospholipid were found to serve as sources of the fatty acids required for cellular phospholipid biosynthesis in the parasite (Holz 1977, Vial et al. 1982, 1984). Fatty acids and phospholipids are essential for membrane synthesis during the parasite development and growth. The malarial parasite, *Plasmodium* spp., is incapable of de novo fatty acid and cholesterol synthesis (Sherman 1979, Vial et al. 1984, Zidovetzki and Sherman 1991). However, *Plasmodium* infected erythrocytes contain four to five times more phospholipids than uninfected erythrocytes (Holz 1977, Sherman 1979). Most of the new phospholipids are believed to be of plasmodial membrane phospholipid origin. Lipids (fatty acids and their metabolites) in *Schistosoma mansoni* are involved in various activities including penetration of skin by cercariae, integral parts of membrane components and antigens, sexual development and resistance to host defenses (see Furlong 1991).

In *P. marinus*, both meront and prezoosporangia stages are characterized by an abundance of refractile bodies, which are lipid droplets. To date little is known about the lipid and fatty acid metabolism of *P. marinus* during development. The elucidation of the metabolism of lipid and fatty acids in the parasite during development and its host/culture medium will give us a better understanding of the lipid metabolism of the parasite.

MATERIALS AND METHODS

P. marinus cells and culture medium:

Meronts were cultured in *P. marinus* culture media according to Gauthier & Vasta (1993) (Media 1) and Kleinschuster and Swink (1993) (Media 2) respectively. *P. marinus* meronts were harvested during their log growth phase. Prezoosporangia were isolated from infected oyster tissue preincubated in thioglycollate medium according to Chu and Greene (1989). To determine the changes in lipid classes in the media and parasite, *P. marinus* cells were incubated in the culture media for a period of one week. After the incubation period, *P. marinus* cells were isolated from the culture medium by centrifugation (800 x g for 10 min), thoroughly washed with filtered sea water (20 ppt) and freeze-dried. Lipid classes and fatty acid composition of *P. marinus* cells and culture media were analyzed as described below. Culture media without *P. marinus* cells were used as controls.

Analysis of lipid classes:

Total lipids were extracted from both the culture media, cultured meronts, prezoosporangia isolated from infected oyster tissue, and thioglycollate medium (thio) with chloroform-methanol-water (2:2:1) according to the procedure described by Bligh and Dyer (1959). One µl of sample was spotted on a silica gel rods (S-III chromarods, Iatron

Laboratories, Tokyo, Japan) and solvent focussed in a 1:1 $CHCl_3$: methanol solution. Separation of lipid classes was achieved by incubation of sample loaded chromarods in a solvent system of hexane:diethyl ether: formic acid (85:15:0:04 v/v/v) for 50 min. After incubation, lipid classes were quantified with flame-ionization (TLC-FID) using an Iatroscan TH-10, MK-3 analyzer (Iatron laboratories). Operating conditions were 2000ml/min air flow, 0.73 kg/cm^3 hydrogen pressure and scan speed of 3.1 mm/sec. Peak area determinations were performed by computer analysis (T DataScan, RSS Inc., Bemis, TN). Quantities of each lipid class were determined by comparison with standard curves of major lipid classes, triacylglycerols (TAGs, menhaden oil), wax/cholesterol esters (WE/CE, oleic acid), free fatty acids (FFA, Stearic acid) cholesterol, and phospholipids (PLs, lecithin) (1, 5, 10 and 20 µg). Standards were analyzed along with the experimental samples. Results were expressed as percentage of total lipid. Total lipid content was expressed as µg lipid/mg of dry cell weight.

Due to the unsatisfactory separation of PLs (polar lipids) on chromarods (Banerjee et al. 1985), separation of PLs was carried out on a high performance thin layer chromatography (HPTLC) plate precoated with silica gel grade G (Whatman), according to Olsen and Henderson (1989). In general, HPTLC plates were activated (180°C for 1 hr) and

spotted with 5 µl of sample in chloroform. The HPTLC plates were then developed in a solvent mixture comprising of methyl acetate: n-propanol: chloroform: methanol: 0.25% aqueous potassium chloride (25:25:25:10:9 v/v/v/v/v). This solvent mixture gave a clear separation of phosphatidylcholine (PC), phosphatidyl-ethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingolipids (SPH) (Vitiello and Zanetta, 1978). The plates were air dried and charred with 3% (w/w) cupric acetate in 8% phosphoric acid at 180°C for 20 min (Fewster et al 1969). The phospholipid classes were then identified and quantified using a transmittance/ reflectance scanning densitometer (Hoefer Scientific Instruments, San Franscisco, CA). Known quantities of purified standards SPH, PC, PS/PI and PE (Sigma Chemical Company, St Louis, MO) were run in parallel along with the experimental samples and response factors of each standard was calculated. Data were corrected using response factors derived from the purified standards and expressed as percentage of total lipid.

Fatty acid analysis:

To determine fatty acid composition, total lipid extracts were transesterified with methanol and borontrifluoride according to Cosper and Ackman (1983). Separation of fatty acid methyl esters (FAMEs) was carried out on a gas chromatograph (GLC Varian 3300) equipped with a flame ionization detector, using a DB-WAX fused silica column capillary column (35m*0.25mm i.d,

J & W Sci). The column temperature was programmed from 120-180°C at 12°C/min and from 180-220°C at 6°C/min. Injector and detector temperatures were 220 and 240°C respectively, and the flow rates of compressed air and hydrogen were 300 and 30 ml/min respectively. Helium was used as carrier gas at 1.5 ml/min. Identification and quantification of FAMEs were based on comparison of the sample retention time to those of known standards. The results were corrected with the response factors of external standards and expressed as % of total FAMEs.

STATISTICAL ANALYSES

A two-way analysis of variance (ANOVA) was used to determine the differences in total lipid and lipid classes in meront incubated culture media 1 and 2 and control media. A one way ANOVA was used to determine differences in lipid classes and fatty acid composition between meronts cultured in both the media; meront and individual culture medium; prezoosporangia and meront (from Medium 2); and between prezoosporangia and thioglycollate medium.

RESULTS

There was a significant decrease ($p < 0.01$) in total lipid content in both *P. marinus* culture Media 1 and 2 respectively,

after 7 days compared to controls. This indicates potential assimilation of lipid by the parasite (Figs 1a and 1b). Culture Medium 2 had significantly higher total and phospholipid content compared to Medium 1 ($p < 0.0001$). In contrast, WE and cholesterol were significantly higher in Medium 1 compared to Medium 2 ($p < 0.001$). The differences in lipid classes between the two culture media (Fig 2a) are reflected in the lipid class pattern of the meronts (Fig 2b). Although differences exist in the lipid class composition of the media and meronts, similar to the media, meronts cultured in Medium 1 had more neutral lipids (TAG) ($p < 0.005$) while meronts cultured in Medium 2 had more PLs (Fig 2b) ($p < 0.05$). Both media had significantly higher % of WE and CHOL, compared to meronts cultured in the same media (Figs 1a, 2 and 3).

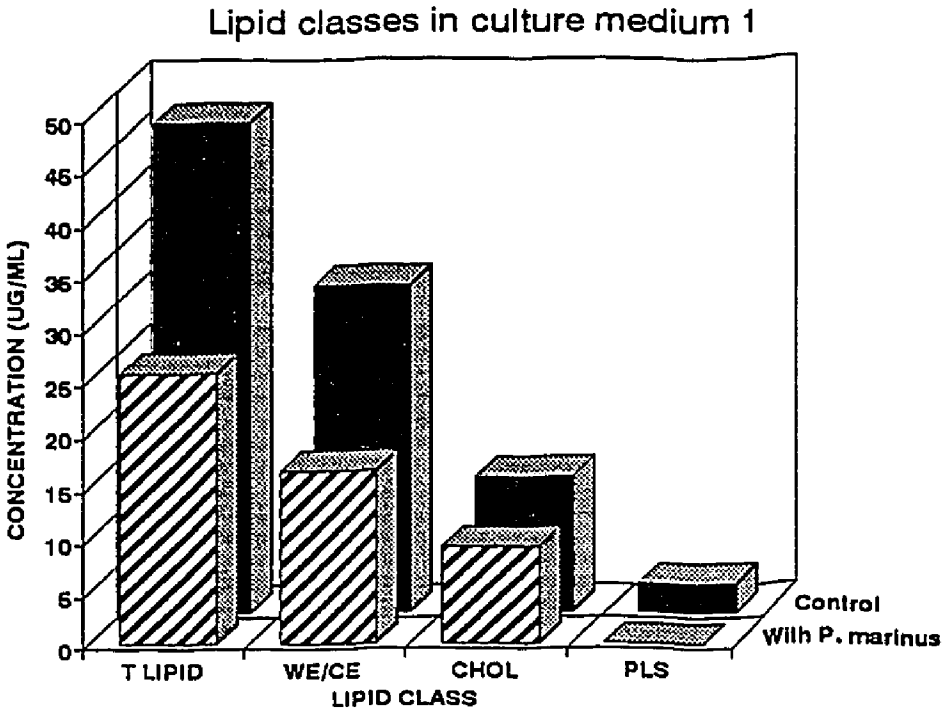
Although, no significant differences in total lipid exist between meronts and prezoosporangia, differences in lipid classes were observed (Fig 4). Prezoosporangia contained more neutral lipids (mostly TAGs) ($p < 0.0001$) compared to meronts, where PLs were the dominant lipid class (Fig 4) ($p < 0.001$). In addition, meronts contained significantly higher WE compared to prezoosporangia ($p < 0.0001$).

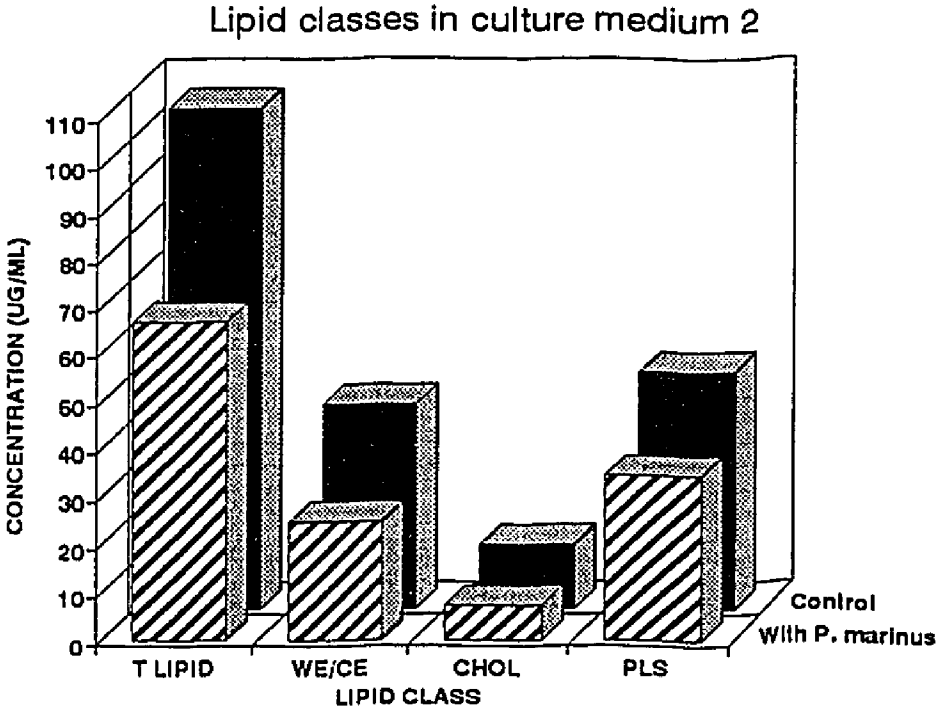
TAGs were the dominant lipid class in prezoosporangia ($p < 0.001$), compared to thioglycollate medium. In comparison, PLs (PS/PI) were the dominant lipid class in thioglycollate

medium and were significantly greater than in prezoosporangia ($p < 0.0001$). In addition, thioglycollate medium had significantly higher WE content compared to prezoosporangia ($p < 0.05$). No PC, SPH, PE and TAG were detected in thioglycollate medium, but were observed in prezoosporangia (Fig 5).

Despite some similarities, significant differences in fatty acid composition (Table 1) exist between *P. marinus* meronts, prezoosporangia and their culture medium. Significant differences also exist between *P. marinus* incubated medium and control medium ($p < 0.05$). Most of the fatty acids in both *P. marinus* cells and their culture medium are long chain fatty acids (> 16 C). Arachidonic acid (20:4n-6) is particularly high in meronts cultured in Medium 2 compared to its culture medium. However, meronts cultured in Medium 1 have a similar percentage to that of their culture medium. The composition of linoleic acid (18:2n-6), linolenic acid (18:3n-3) and docosahexanoic acid (22:6n-3) was higher in both the culture media compared to the meronts. However, prezoosporangia had higher DHA content compared to thioglycollate medium. Meronts cultured in either media had high content of 20:1 and 20:2, despite lacking in the culture medium.

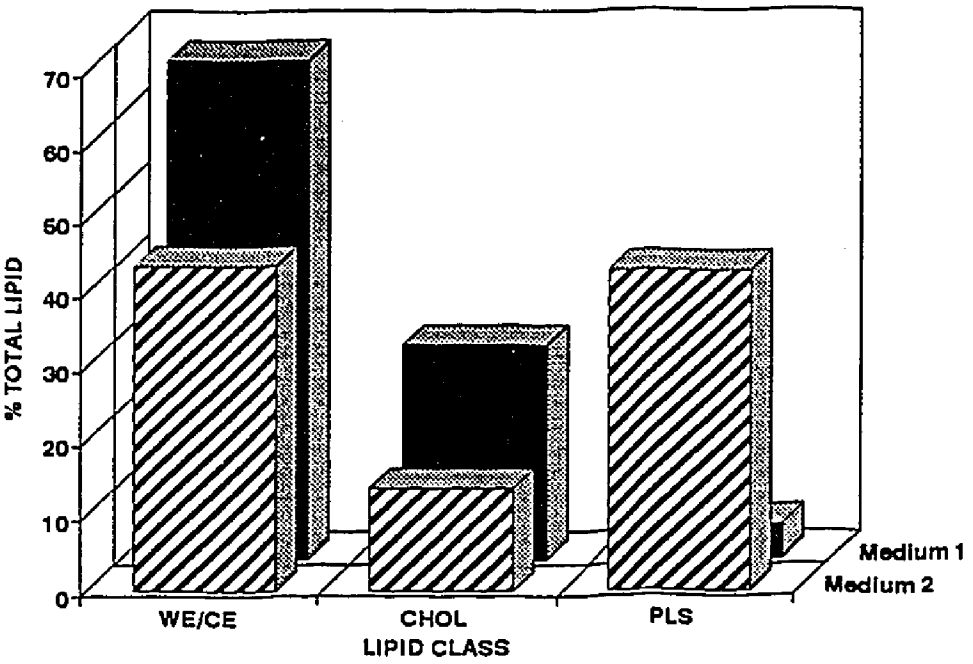
Figs 1a and 1b: Lipid class composition of culture media after 1 week of incubation with *P. marinus* meronts. Fig 1a: media 1; Fig 1b: Media 2. T lipid = total lipid /ml of media, WE/CE = Wax/cholesterol esters, CHOL = cholesterol, PLs = phospholipids.


Lipid classes in culture medium 1
CONCENTRATION (UG/ML)
50
45
40
35
30
25
20
15
10
5
0
T LIPID
WE/CE
CHOL
PLS
LIPID CLASS
Control
With P. marinus


Lipid classes in culture medium 2
CONCENTRATION (UG/ML)
110
100
90
80
70
60
50
40
30
20
10
0
T LIPID
WE/CE
CHOL
PLS
LIPID CLASS
Control
With P. marinus

Figs 2a and 2b: Lipid class composition of culture media 1 and 2 (Fig 2a) and meronts cultured in respective media (Fig 2b). WE/CE = Wax/cholesterol esters, TAG = triacylglycerol, CHOL = cholesterol, PLs = phospholipids.

Lipid classes in media 1 and 2



Lipid classes in meronts

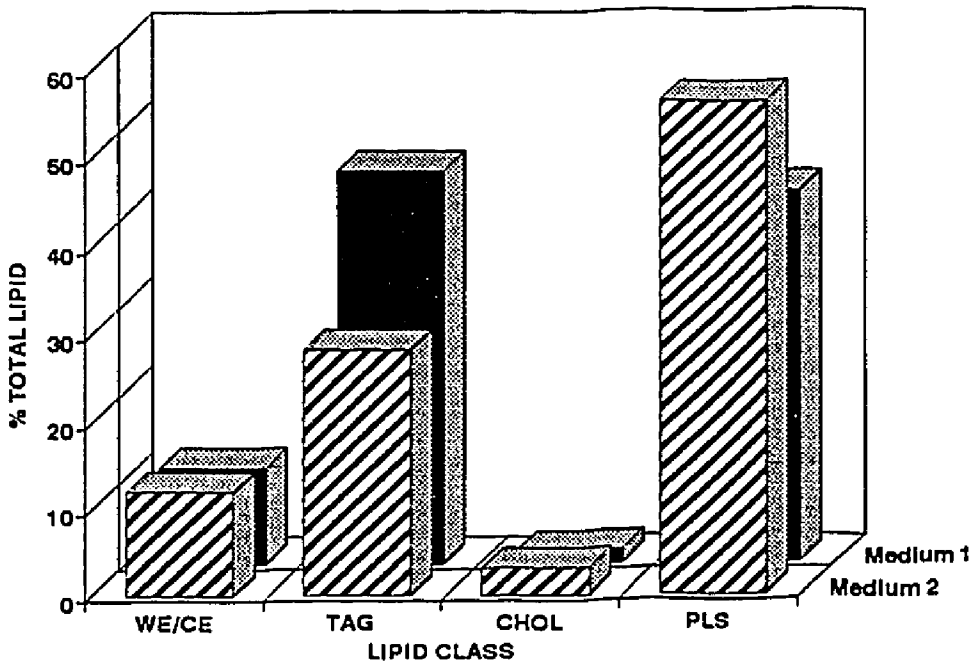


Fig 3: Comparison of lipid class composition of media 2 and meronts cultured in the same medium. WE/CE = Wax/cholesterol esters, TAG = triacylglycerol, CHOL = cholesterol, SPH = sphingolipids, PC = phosphatidylcholine, PS/PI = phosphatidylserine and phosphatidylinositol, PE = phosphatidylethanolamine.

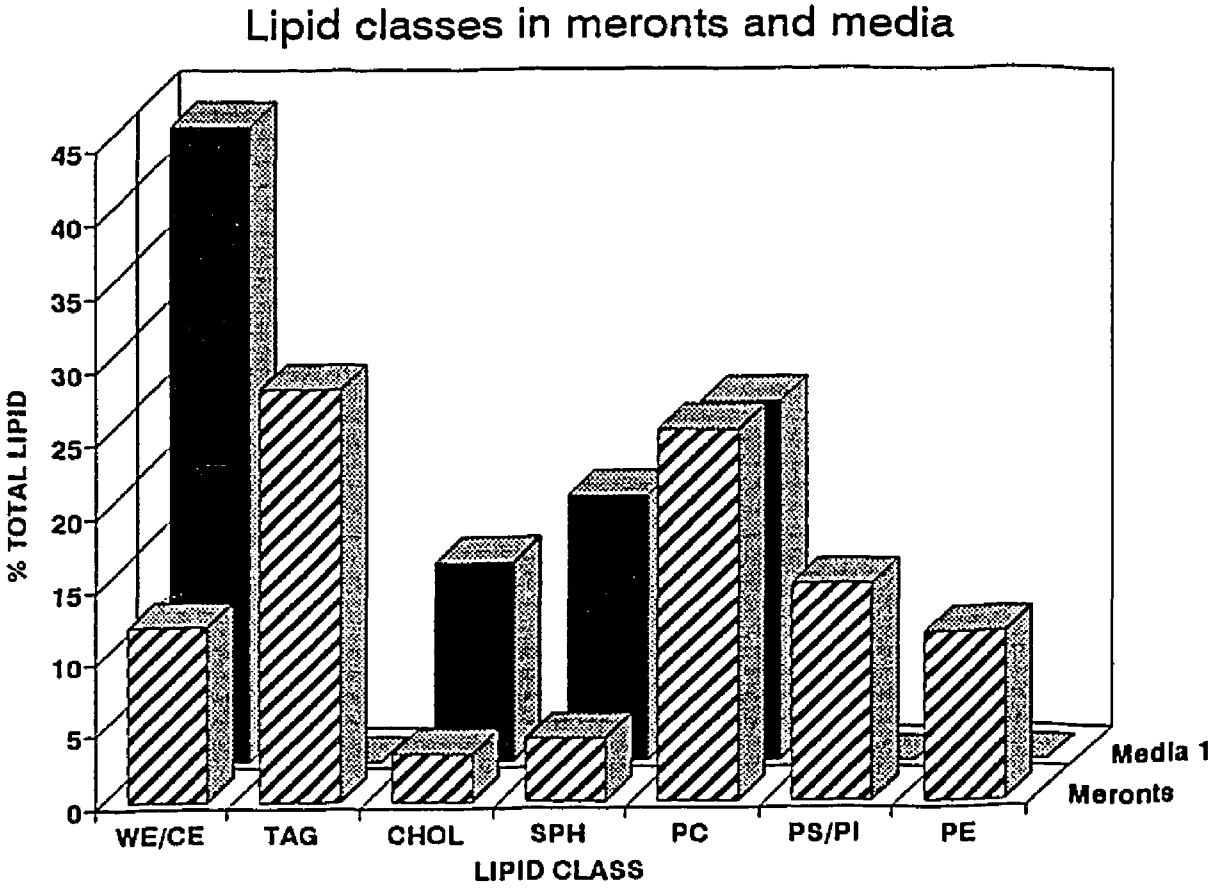

Lipid classes in meronts and media
% TOTAL LIPID
45
40
35
30
25
20
15
10
5
0
WE/CE
TAG
CHOL
SPH
PC
PS/PI
PE
LIPID CLASS
Media 1
Meronts

Fig 4: Comparison of lipid class composition of meronts cultured in media 2 and prezoosporangia isolated from infected oyster tissue. WE/CE = Wax/cholesterol esters, TAG = triacylglycerol, CHOL = cholesterol, SPH = sphingolipids, PC = phosphatidylcholine, PS/PI = phosphatidylserine, phosphatidylinositol, PE = phosphatidylethanolamine.

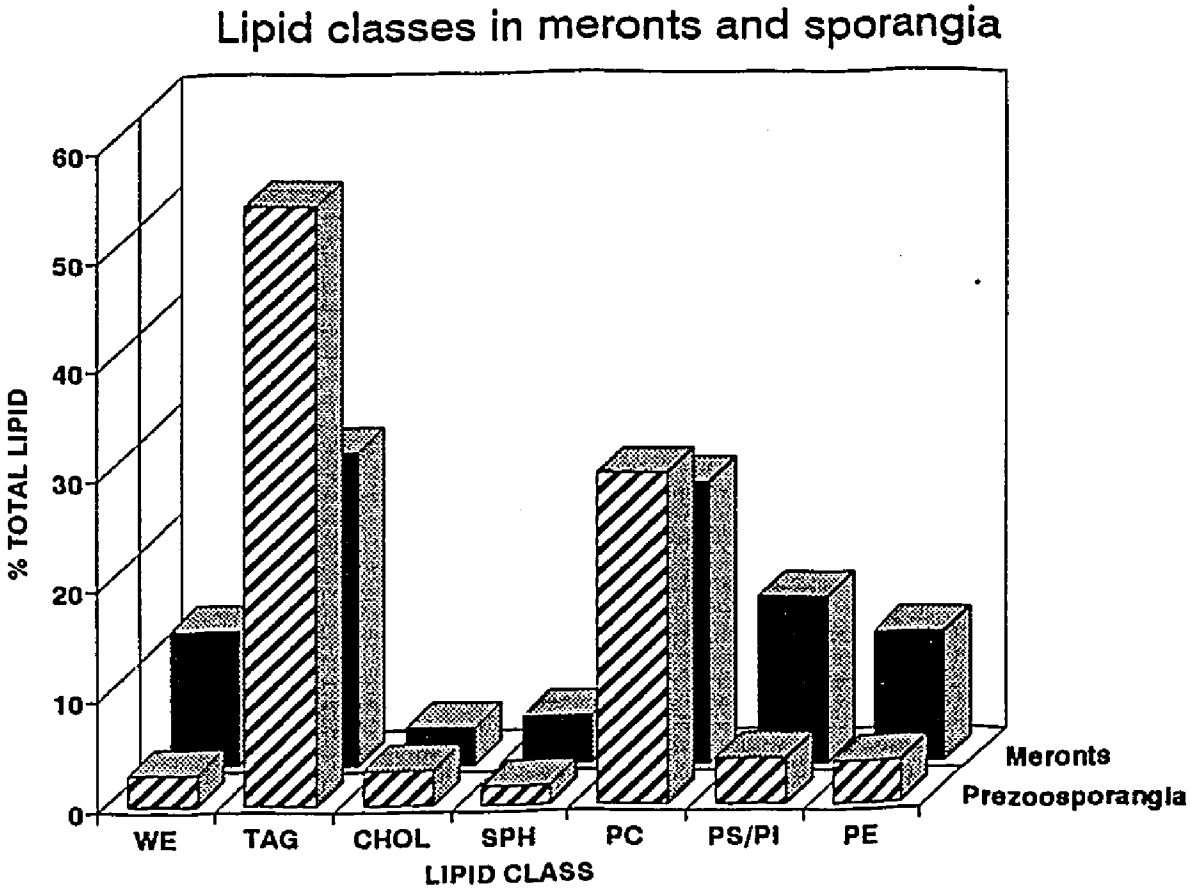

Lipid classes in meronts and sporangia
% TOTAL LIPID
60
50
40
30
20
10
0
WE
TAG
CHOL
SPH
PC
PS/PI
PE
LIPID CLASS
Meronts
Prezoosporangia

Fig 5: Comparison of lipid class composition of prezoosporangia isolated from infected oyster tissue and thioglycollate medium. WE/CE = Wax/cholesterol esters, TAG = triacylglycerol, CHOL = cholesterol, SPH = sphingolipids, PC = phosphatidylcholine, PS/PI = phosphatidylserine, phosphatidylinositol, PE = phosphatidylethanolamine.

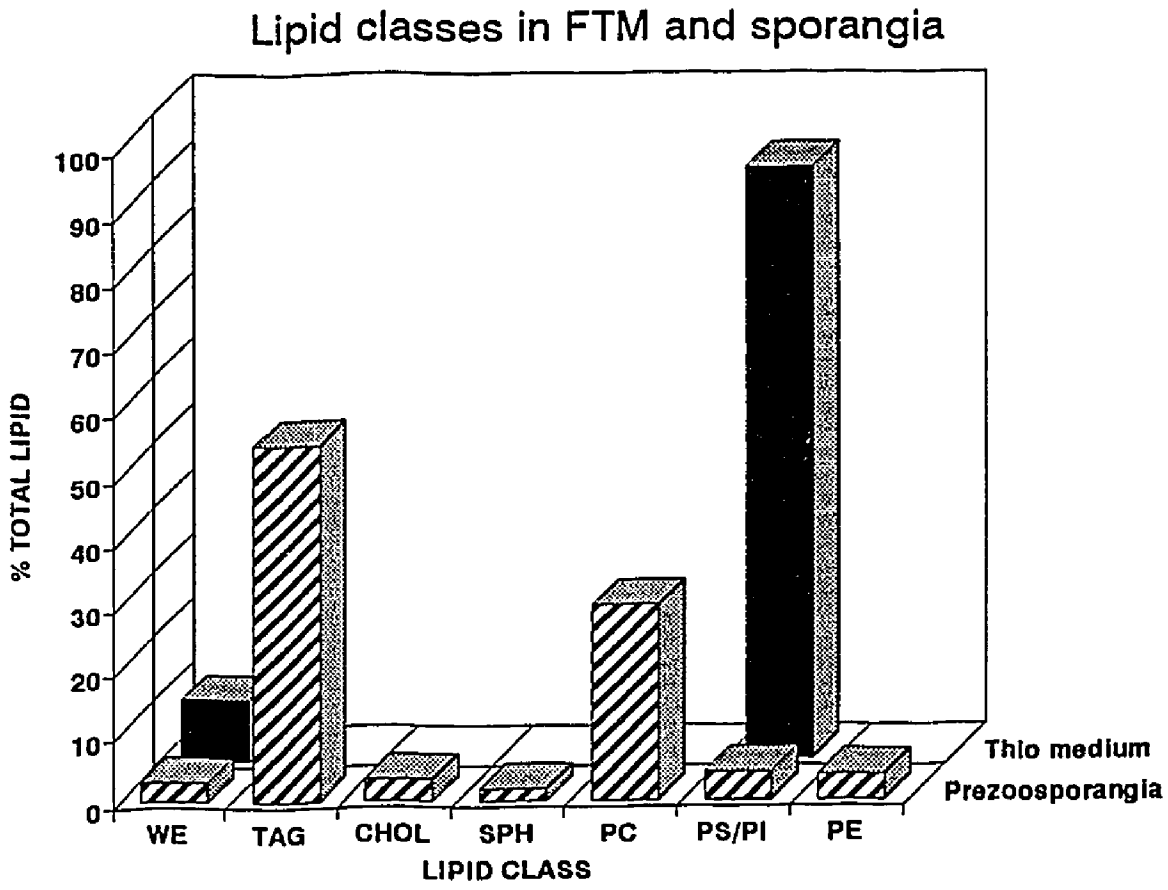

Lipid classes in FTM and sporangia
% TOTAL LIPID
100
90
80
70
60
50
40
30
20
10
0
WE
TAG
CHOL
SPH
PC
PS/PI
PE
LIPID CLASS
Thio medium
Prezoosporangia

TABLE 1: Lipid and fatty acid composition of *P. marinus* culture media 1 and 2 (test and control), meronts, thioglycollate medium (thio) and prezoosporangia.

Fatty acid composition

Fatty acid	Medium 1			Medium 2				
	Control medium	Medium with *P. marinus*	Meront	Control medium	Medium with *P. marinus*	Meront	Thio	Prezoosporangia
12:0	0	10.85	6.59	10.22[a]	21.58[a]	1.38[b]	-	-
14:0	0.65[a]	1.07[a]	6.36[b]	0.45[a]	1.29[b]	3.99[c]	0.74[a]	1.85[b]
15:0	0.43	0.84	0.04	0.33	0.66	0.26	0.82	0.64
16:0	18.88	13.15	14.55	15.89	13.86	14.22	7.05	9.55
16:1n-7	2.57	0.73	1.56	2.63	2.27	1.25	1.05	4.27
16:1	-	-	-	-	-	-	-	-
16:2	0.05	-	0.02	0.17	-	-	-	3.05
16:3	0.14	-	-	0.18	-	-	1.21	0.83
16:4	0.81	-	0.01	0.79	0.44	0.14	-	0.67
17:0	0.60	0.15	-	0.45	-	0.27	-	0.32
18:0	10.64	7.65	7.19	7.97[a]	3.82[b]	7.32[a]	5.08[a]	1.00[b]
18:1n-9	13.38	11.16	14.1	13.80	10.85	13.76	-	0.67
18:1n-7	8.74[a]	3.12[b]	4.13[b]	6.42[a]	4.27[b]	3.58[b]	14.30[a]	2.18[b]
18:2n-6	4.51[a]	1.38[b]	1.42[b]	3.78[a]	3.22[a]	1.62[b]	1.84	1.32
18:3n-3	-	-	0.02	0.17[a]	0.93[b]	0.19[a]	0.56	1.79
18:4n-3	0.85	0.99	-	0.38	0.97	-	1.43	5.24
20:0	0.25[a]	0.40[b]	1.90[c]	0.12[a]	0.06[a]	1.97[b]	0.31	0.30
20:1	0.15[a]	1.27[b]	13.94[c]	0.14[a]	0.74[b]	15.18[c]	0.08[a]	0.61[b]
20:2	1.48[a]	1.43[a]	2.17[b]	0.66	1.48	5.13	2.18[a]	0.31[b]
20:3	1.31	0.05	0.55	1.15[a]	0.41[b]	0.54[b]	0.10	0.03
20:4n-6	8.18[a]	1.79[b]	8.66[a]	6.14[a]	4.70[b]	13.68[c]	-	7.20
20:4n-3	4.70	0.89	-	0.23	0.43	-	1.71	1.95
20:5n-3	0.27	0.42	0.13	0.59[a]	0.34[a]	0.17[b]	1.47	1.46
22:0	0.12[a]	0.67[a]	0.86[b]	0.20[a]	0.22[a]	1.12[b]	2.06[a]	26.45[b]
22:1	-	0.40	0.42	0.01[a]	0.08[a]	1.10[b]	16.90[a]	0.07[b]
22:5n-3	1.16[a]	1.51[a]	2.17[b]	1.39[a]	1.54[a]	3.53[b]	0.37	0.69
22:6n-3	4.62[a]	0.37[b]	0.59[b]	2.48[a]	2.20[a]	0.79[b]	0.56[a]	16.13[b]

DISCUSSION

Results of the present study (Figs 1a and 1b) indicate that there was an uptake of lipid classes by *P. marinus* meronts from culture media. Presence of phospholipases has been demonstrated in other protozoan parasites such as the malarial parasite, *Plasmodium* spp (Zidovetzki et al. 1993). Lipases or phospholipases may aid the parasite by breaking down the host lipids and facilitate the uptake of resulting fatty acids. Preliminary studies in our laboratory indicate that *P. marinus* posesses lipolytic enzymes, lipase, and phospholipase A_2.

Results, suggest that *P. marinus* may be able to convert WE/CE into TAGs and phospholipids, such as phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine (Fig 3a). Although, TAGs were absent in Medium 2, TAGs were present in meronts cultured in this medium. Similarly, PLs, PS/PI and PE, absent in the medium, were observed in the meronts. The abundance of WE and CHOL in Medium 2 compared to meronts cultured in the same medium indicate that differences exist between the lipid class composition of the parasite and the medium. These results may suggest that the parasite selectively obtains lipid classes and converts them into its constituent lipid components. Cholesterol is the primary precursor of steroids which are known to regulate development and growth in marine organisms (Kanazawa et al. 1975, Goad

1976). Cholesterol was observed in both meronts and prozoosporangia (Figs 2b, 3, and 4). Synthesis of cholesterol *de novo* by parasites has not been reported before. *P. marinus* may be obtaining cholesterol from the culture medium and may utilize it in the parasites' metabolism. As mentioned earlier, the malarial parasite, *Plasmodium* spp, is incapable of de novo fatty acid and cholesterol synthesis (Sherman 1979, Vial et al. 1984, Zidovetzki and Sherman 1991) which are essential for membrane synthesis during the parasites' development and growth. The absolute requirement of the intracellular parasite for host fatty acids has led to suggestions that plasmodial phospholipid metabolism could be a chemotherapeutic target (Vial et al. 1982). Similarly, the dominance of PLs in meronts may be an indication of active assimilation or synthesis of PLs using lipid from the host as a source (in this case, culture medium) for membrane formation in the rapidly proliferating cells. This result is not surprising since the growth rate of *P. marinus* meronts in culture is 0.7-1 doubling per day. However, it is not known if *P. marinus* can synthesize ceratin fatty acids required for membrane synthesis. As indicated by the decrease in total lipid content in *P. marinus* culture medium compared control medium, *P. marinus* may be obtaining lipids from the culture medium/host to fulfill its nutritional and metabolic requirements. Similarly, prezoosporangia appear to use phospholipids taken up from the fluid thioglycollate medium

into neutral lipids as reserves (Fig 3b). Presumably similar mechanisms may be taking place while the parasite is in the host. Alternatively, prezoosporangia may have obtained neutral lipids (Fig 3b) from the host, oyster tissue. Prezoosporangia are usually seen in near dying or morbid oysters. Thus, when the oyster dies, tissue disintegrates and prezoosporangia are released into the water. The parasite may use TAGs, the usual form of storage lipids, as energy reserve for further development upon release into the water column.

Differences in fatty acid composition were noted in different lifestages of the tapeworm *S. erinacei* (Fukushima et al 1988). The proportion of the fatty acid composition in mature and plerocercoid stages of *S. erinacei* closely resembled the host tissue and serum composition. However, in the present study, although the fatty acid composition of meronts and prezoosporangia closely resembled their culture media, some differences in fatty acid composition were noted between *P. marinus* cells and media (Table 1). The presence of 20:1 and 20:2 fatty acids in meronts, despite their absence in the culture medium, suggests that *P. marinus* preferentially takes up certain fatty acids and/or elongates and desaturates short chain fatty acids of host. Biosynthesis and interconversion of fatty acids was demonstrated in the cestode parasite, *H. diminuta* by Jacobsen and Fairbairn (1967). Also, differences in fatty acid composition of the phospholipid

classes in the fish parasite, *Paratenuisentis ambiguus* and its' host, *Anguilla anguilla* were demonstrated earlier (Aitzetmuller et al. 1994). Arachidonic acid is the primary precursor of many important biochemical components including prostaglandins (Fusco et al. 1985). The higher % of arachidonic acid in *P. marinus* (7-13.7%) compared to host oyster (<3%) (Chu et al. 1990), may play a role in the synthesis of prostaglandins which control and regulate growth of the parasite. Secretion of eicosanoids by *Scistosoma* spp, aid them in penetration of the host tissue by initiating cycloxygenase system (Golan et al. 1986). Eicosanoids are metabolites of essential fatty acids. *S. mansoni* produces a lysophospholipid, monopalmitoylphosphatidylcholine, which has detergent like properties. This may aid the parasite in obtaining lipids from host cell membranes (Furlong 1991), including cholesterol (Golan et al. 1988). Lysophosphatidylcholine from *Schistosoma* spp. lyses human red blood cells, thus compromising the defense functions of the host (Golan et al. 1986). Although, lysophospholipids have not been observed in *P. marinus* cells in the present study, their existance or production by *P. marinus* cannot be ruled out. Lysophospholipids may be produced upon interaction with the host tissue.

In summary, *P. marinus* cells may be capable of obtaining lipids from the host source and the lipid class composition of

the parasite depends on the source of lipids. *P. marinus* cells may be capable of limited elongation and desaturation of host fatty acids and preferentially retain certain fatty acids (eg arachidonic acid). Future studies should investigate the de novo synthesis of fatty acids by *P. marinus*. The differences in fatty acid composition between meronts and prezoosporangia should be further investigated before targetting them as potential targets for chemotherapy.

CHAPTER 7

CONCLUSIONS AND SUMMARY

The following summarizes the major findings of this study:

1. Both meront and prezoosporangia stages of *P. marinus* are infective, with meronts being more infective than prezoosporangia under laboratory conditions. Moderate to heavy *P. marinus* infection in oysters reduces the condition index of oysters. Oysters infected with meronts showed reduction of plasma lysozyme compared to uninfected oysters. Oysters infected with prezoosporangia showed a reduction in plasma protein concentration.

2. There is a dose dependent relationship between the number of infective particles encountered by the oyster and disease prevalence and intensity. The minimum number of *P. marinus* cells required to initiate infection in oysters is 10-100 cells.

3. Although, temperature, salinity and dose of *P. marinus* cells by themselves are important, their interaction does not significantly effect prevalence of *P. marinus* infection in oysters. However, the interaction between temperature and dose and between temperature and salinity are significant in influencing the intensity of infection.

4. Temperature and salinity significantly effect cellular and

humoral factors. Environmental history including the genetic factors of oysters may also play a role in this context.

5. *P. marinus* suppresses reactive oxygen intermediate (ROI) production by oysters. Among the suite of antioxidant enzymes in *P. marinus* examined, only acid phosphatase was detected. Acid phosphatase secreted by the parasite may be acting as an antioxidant enzyme. Both extra- and intracellular acid phosphatase activities of *P. marinus* are higher than its host and activity increases with increasing temperature and salinity.

6. The acid phosphatase secretion by *in vitro* cultured *P. marinus* is dependent on the dose of cells. Growth of *P. marinus* is positively related to temperature and salinity (osmolality). Acid phosphatase may aid in parasites' nutrition and defense against the host. The localization of acid phosphatase in the nucleus of the parasite may suggest that the enzyme may also be involved in cell cycle regulation.

7. Lipid and fatty acid composition of *P. marinus* cell stages differs significantly from each other and is also influenced by the composition of the culture medium. Results also suggest that *P. marinus* may be able to convert wax esters / cholesterol esters into other lipid classes. *P. marinus* may

actively assimilate arachidonic acid and/or modify short chain fatty acids from the host.

In nature, host-parasite interactions are quite complex and involve host factors such as age, nutrition, reproductive status, genetics, and immunocompetence, and parasite factors such as virulence, genetics, and physiology. The interaction of these factors in turn are regulated by environmental factors (e.g. temperature, salinity etc). The environmental history (eg. temperature and salinity of the habitat) of the oyster populations may also play a strong role in determining the host factors. The interaction of environmental, host and parasite factors governs the balance either in favor of the host or the parasite.

In this chapter, I will discuss the relationship of environmental, host, and parasite factors relevant to my findings.

Results of Chapter 1 indicate that both meronts and prezoosporangia are infective, with meronts being more infective. Although, biflagellated-zoospores were not included in my study for comparison, I feel that biflagellated-zoospores may not be as effective as the meront stage for disease transmission due to the following reasons: 1) No biflagellated-zoospores were observed in nature and in

the tissues of dying oysters; 2) Recent attempts to induce zoosporulation in prezoosporangia were unsuccessful. However, it has been shown that biflagellated-zoospores can induce infections (Chu et al., unpublished results; Roberts et. al., unpublished results). The higher infection prevalence and intensity in meront-challenged oysters may be due to the rapid proliferation rate of meronts at higher temperatures and salinities. In addition, culture of prezoosporangia in thioglycollate medium may have affected their infectivity and/or viability after inoculation in oyster tissue. Oysters challenged with cultured meronts resulted in lower infection intensities compared to oysters challenged with comparable dose of meronts isolated from infected oyster tissues incubated in thioglycollate medium (Volety and Chu, unpublished results, Perkins, personal communication). Results of the present study suggest that meronts may be the primary infective lifestage of *P. marinus* (Perkins 1988). Meronts induced greater infection prevalences and intensities, but, prezoosporangia-infected oysters showed a lower condition index and protein concentration than meront-infected oysters. This suggests that prezoosporangia may exert a higher energetic demand on the host than do meronts. Meront-infected oysters showed a significantly-lower lysozyme activity than uninfected oysters (Chapter 1). However, no differences in lysozyme activity were observed between meront- and prezoosporangia-infected oysters; and between prezoosporangia

infected oysters and uninfected oysters.

Results of Chapter 2 confirm that meronts are more infective than prezoosporangia. The prevalence and intensity of infection in oysters is dependent on the dose of infective particles encountered by the oyster. As few as 10-100 *P. marinus* cells are required to initiate infection in oysters. These results support Mackin's (1962) findings that infection in oysters depends on the dose of *P. marinus* cells. Temperature was the most important factor influencing *P. marinus* prevalence in oysters, followed by infective cell dose and salinity respectively. Although temperature, salinity and infective cell dose by themselves significantly affect the prevalence of *P. marinus* infection, the interaction of these three factors, does not affect the prevalence. However, the interaction between temperature and salinity; and, between temperature and infective cell dose, significantly intensifies the disease in oysters. It has been suggested by Scott et al. (1985) that salinity effects the physiology of the host and the parasite, and thus the outcome of the infection. Results also indicate that temperature and salinity complicated by the environmental history or genetic differences of oysters significantly affect the condition index, cellular and humoral factors in oysters. Despite the gradual acclimation, the high mortality of oysters at 3 ppt and 25°C indicates that oysters from Maine, which were used to living in a high salinity and

low temperature water were severely stressed at high temperatures and low salinities. The lowered condition index of oysters at higher temperatures suggests that temperature stresses the oysters, possibly by increasing their metabolic rates. Infected oysters also showed significantly lower condition index than uninfected oysters. This may suggest that infection negatively affects the physiological status of the oyster.

In contrast to previous findings reported by Chu and La Peyre (1993a), the total hemocyte number in oysters were higher at 10 and 15°C than at 25°C in the present study. No significant differences in total hemocytes and percentage of granulocytes were observed between infected and uninfected oysters in the present study. However, earlier studies revealed that there was a significant increase in higher hemocyte number in oysters infected with *P. marinus* (Chu and La Peyre 1993a), and *H. nelsoni* (Ling 1990, Ford et al. 1993). The higher total hemocyte number and percentage of granulocytes at higher temperatures and salinities in these previous studies may be due to temperature, salinity and pathological effects. In addition, higher prevalences of infection were observed at elevated temperatures and salinities in all these studies. Based on these observations, a protective or a potential biomarker role cannot be assigned to either total hemocyte number or percentage of granulocytes.

Apparently, total hemocyte number in oysters is affected by a number of factors, for example, heart rate (which in turn is influenced by temperature) (Feng 1965), wounding (Pauley and Sparks 1965), feeding (Feng et al. 1971), and infection (Ford et al. 1993).

Higher lysozyme concentration in oysters at 20 ppt than at 10 and 3 ppt in the present study is in contrast to earlier findings (Chu et al. 1993). Lysozyme concentration was higher in oysters at low salinities than at high salinities (Chu et al. 1993). It must be noted, however, that the oysters used in the present study were obtained from an area of consistently high salinity. The earlier studies conducted by Chu et al. (1993) which used oysters from low salinity areas such as the Rappahannock River and James River. No significant differences in lysozyme concentration were noted between infected and uninfected oysters in the present study. This result is consistent with the findings of previous temperature and salinity effect studies (Chu et al. 1993, Chu and La Peyre 1993a). However, a negative correlation was observed between lysozyme concentration and temperature, salinity and *P. marinus* infection in oysters (Chu and La Peyre 1993, Chu et al. 1993). Since the uninfected oyster group (Chapter 2) included data from control (non-*P. marinus*) group oysters, it is difficult to say, at the present time, that higher lysozyme activity in oysters did not result in lowered

infection or resistance against *P. marinus* by the oysters and hence no conclusions can be made regarding the "protective role" of lysozyme(s) in oysters against *P. marinus*. The exact role of oyster lysozyme in host defense against *P. marinus* need to be verified. The observed changes in total hemocyte number, percentage of granulocytes, protein and lysozyme concentration in the present study may only be due to the effect of environmental factors in addition to environmental history or genetic factors and may not be involved in resistance.

Results of chapter 3 clearly demonstrated that *P. marinus* is capable of suppressing ROI production of oyster hemocytes. Parasites such as *Leishmania* spp., *Toxoplasma* spp., and *Trypanosoma* spp. are capable of being phagocytosed without stimulating a respiratory burst. These parasites escape superoxide-dependent killing by secreting anti-oxidant enzymes (e.g. catalase, superoxide dismutase, acid phosphatase, glutathione peroxidase etc) (Weiss et al. 1987, Remaley et al. 1984, Le Gall et al. 1991, and Mkoji et al. 1988a, 1988b). Oyster hemocytes can recognize and phagocytose *P. marinus* meronts with limited degradation of the parasite in the phagocytic vacuole (La Peyre 1993, Bushek 1994). Antioxidant enzymes inhibit and/or scavenge the ROI produced by the host phagocytes (Mauel 1984, Bogdan et al. 1990). *P. marinus* may posses anti-oxidant enzymes or enzymes responsible for

suppressing the ROI production of hemocytes.

These results suggest that: 1) either lysosomal enzymes are not very effective in degrading *P. marinus* meronts; and/or 2) meronts are capable of suppressing the respiratory burst of oyster hemocytes. Presence of acid phosphatase in the extracellular medium, and failure to detect other antioxidant enzymes suggest that acid phosphatase(s) may be acting as an antioxidant enzyme, possibly by dephosphorylating the enzymes responsible for ROI production. Acid phosphatase from *Leishmania* spp., and rickettisiales-like organisms inhibits the ROI production from their hosts (Remaley et al. 1984, Le Gall et al. 1991.). Also, the presence of acid phosphatase in the nucleus of P. marinus may suggest that acid phosphatase may be involved in functions such as cell cycle regulation. As mentioned earlier (Chapter 4), involvement of phosphatases in signalling interferon γ and signal transduction (Walton and Dixon 1993), DNA synthesis (Brautigan 1992) and cell cycle regulation (Freeman and Donoghue 1991) have been described. Phosphatases and kinases activate and deactivate each other, thereby regulating the cell cycle events (Meek and Street 1992).

The reasons for the difference in acid phosphatase activity between oysters from Chesapeake Bay and Maine are not known at the present time. Differences in genetic variation,

food, and habitat etc. may be some of the reasons for this difference in acid phosphatase activity. It is tempting to speculate that the higher AP activity in meronts compared to prezoosporangiae may be one of the reasons for the greater virulence of meronts.

It has been suggested that phosphotyrosine phosphatases in bacteria of the genus, *Yersinia* spp. was responsible for its virulence (see Walton and Dixon 1993). Through the present study (Chapters 3 and 4), it was found that *P. marinus* secretes acid phosphatase extracellularly. Studies by Katakura and Kobayashi (1988) indicated that the virulent clones of *L. donavani* secrete acid phosphatase (which migrates slowly during electrophoretic separation, compared to a faster migrating band present in both virulent and avirulent clones) which is not secreted by avirulent clones. In addition, virulent cells produced relatively high levels of acid phosphatase during their growth in culture compared to avirulent cells (Katakura and Kobayashi 1988). It is interesting to note that acid phosphatase secretion increases with increase in temperature and salinity (Chapter 4) and the trend is similar to the increased infections in oysters at elevated temperatures and salinities. Apparently, temperature and salinity affect the physiology of *P. marinus*. Protease secretion activity of *P. marinus* also increases with the increase of temperature and salinity (Garries et al. 1994).

Similarly, *in vitro* studies by Chu and Greene (1989) and Burreson et al. (1994) indicate that temperature and salinity affect the viability of *P. marinus* cells. Temperatures below 4°C and salinities below 6 ppt prevent zoosporulation of sporangia in vitro (Chu and Greene 1989). The higher mortality of meronts occurred at low salinities. It is speculated that low salinities effect the cell volume regulation ability of the parasite (Burreson et al. 1994).

Lipids play a unique role in parasite growth and development (Furlong 1991). Interesting observations were made from the characterization of lipid and fatty acid composition of *P. marinus* (Chapter 6). *P. marinus* may be able to convert wax and cholesterol esters into other lipid classes such as triacylglycerols, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine (chapter 6). Lipids in parasites are involved in a multitude of functions, apart from being energy reserves (see Furlong 1991). Lipids in the helminth parasite, *S. mansoni* were suggested to be involved not only in maintenance of surface integrity and structural requirements, but also for egg production, and cell-cell signalling, and avoidance of host defense. Assimilation of arachidonic acid by *P. marinus* in higher percentages (7-14%) compared to host (<2%, Chu et al. 1990), leads to more interesting questions regarding its role in avoidance of host defense. It is known that eicosanoids are

involved in cell penetration, and host cell damage (Golan et al. 1986).

In summary, *P. marinus* meronts with faster growth rates may be the principal agents for *P. marinus* disease transmission in oysters. Higher temperatures and salinities significantly affect the humoral and cellular factors. However, the responses are different between oyster populations (e.g. Chesapeake Bay and Maine oysters) and may be governed by their environmental and genetic factors. Higher growth of *P. marinus* at warm temperatures (> 15°C) and higher salinities (> 10 ppt) favor *P. marinus*, resulting in the production of acid phosphatase(s) and/or extracellular secretions which may be involved in nutrition and suppression of oxygen metabolite production by hemocytes. Suppression may occur as a result of dephosphorylating the enzymes responsible for ROI production. Lower temperatures and salinities negatively affect *P. marinus* metabolic or growth rate and possibly osmoregulatory functions, thus affecting their survival in oysters. Lipids appear to play a role in parasite nutrition, but no defense function could be attributed to *P. marinus* lipids at this time. The location of acid phosphatase in the nucleus and high assimilation of arachidonic acid by the parasite, relative to the host should be investigated further.

Future studies:

Extensive studies regarding *P. marinus* disease processes in the recent years have contributed to better understanding of *P. marinus*-oyster interactions. Future studies should involve purification and characterization of acid phosphatase and test its role in the parasites' virulence. Investigations should also examine if any differences exist in acid phosphatase secretion by different strains of *P. marinus* and if the extracellular acid phosphatase is correlated with the parasites' infectivity. In addition, efforts should be directed to examine the lipid metabolism of the parasite relative to the host. The differences in metabolic pathways between the host and the parasite may then be exploited for chemotherapeutic purposes.

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